

Complementary Activity of ETV5, RBPJ and TCF3 Drives Formative Transition from Naïve Pluripotency

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SUMMARY

The gene regulatory network (GRN) of naïve mouse embryonic stem cells (ESC) must be reconfigured to enable lineage commitment. TCF3 sanctions rewiring by suppressing components of the ESC transcription factor circuitry. However, TCF3 depletion only delays, and does not prevent, transition to formative pluripotency. Here we delineate additional contributions of the ETS-family transcription factor ETV5 and the repressor RBPJ. In response to ERK signalling, ETV5 switches activity from supporting self-renewal and undergoes genome relocation linked to commissioning of enhancers activated in formative epiblast. Independent up-regulation of RBPJ prevents re-expression of potent naïve factors, TBX3 and NANOG, to secure exit from the naïve state. Triple deletion of *Etv5*, *Rbpj* and *Tcf3* disables

ESC, such that they remain largely undifferentiated and locked in self-renewal even in the presence of differentiation stimuli. Thus genetic elimination of three complementary drivers of network transition stalls developmental progression, emulating environmental insulation by small molecule inhibitors.

INTRODUCTION

Mouse embryonic stem cells (ESC) are in vitro cell lines that retain a high degree of molecular and functional correspondence with the naïve pluripotent epiblast of the pre-implantation embryo (Boroviak et al., 2014; Bradley et al., 1984; Evans and Kaufman, 1981; Martin, 1981). Accordingly, they provide a rich resource for studying mechanisms underlying developmental decisions and transitions. In particular, the ESC pathway to differentiation in vitro provides an opportunity to dissect the progression of pluripotency from naïve founder cells through to specification of germline and somatic lineage progenitors.

Culture in the presence of two small molecule inhibitors (2i) that suppress the MEK/Erk pathway and glycogen synthase kinase-3 (GSK3) sustains stable expression of transcription factor components of the naïve pluripotency gene regulatory network (GRN) (Dunn et al., 2014; Wray et al., 2010; Ying et al., 2008). ESC in these serum-free conditions are proposed to reside in a regulatory ground state (Ying et al., 2008). Upon release from 2i, ESC transition into a distinct second stage of pluripotency that we have termed “formative” (Kalkan and Smith, 2014; Smith, 2017). Formative pluripotent cells have lost GRN components diagnostic of naïve pluripotency and gained transcription factors characteristic of the peri-implantation epiblast such as POU3f1, OTX2, and LEF1. Functional ESC identity is extinguished concomitant with change in transcription factor complement (Kalkan et al., 2017). In parallel, epigenetic processes such as DNA methylation are up-regulated, and competence is gained for lineage induction (Hayashi et al., 2011; Mulas et al., 2017) and onward progression to primed pluripotency. The naïve to formative conversion in a simple and well-defined culture environment simulates events in the peri-implantation mouse embryo (Kalkan et al., 2017), and provides a sensitised platform for identifying factors and mechanisms that mediate change in cell identity (Buecker et al., 2014; Kalkan and Smith, 2014).

Genetic screens have identified several genes that promote ESC transition (Betschinger et al., 2013; Leeb et al., 2014; Li et al., 2018; Villegas et al., 2018; Yang et al., 2012). TCF3 (gene name *Tcf7l1*) was the first factor identified (Guo et al., 2011b) and is recurrently recovered. TCF3 represses key naïve transcription factors ESRRB, TFCP2L1, NANOG and KLF2 (Martello et al., 2012; Pereira et al., 2006), an effect blocked by GSK3 inhibition in 2i culture (Wray et al., 2011). Other pathways and factors identified in the screens have also been shown to reduce expression or function of components of the naïve GRN, although to a lesser extent than *Tcf3*. Strikingly, the majority of these components are present in naïve ESC but are ineffective in 2i (Kalkan and Smith, 2014). The pre-existence of multi-layered dissolution machinery means that mouse ESC are poised for rapid disabling of the naïve network. Multiple effectors also explain why single factor mutants only delay and do not prevent transition.

However, while elimination of naïve factors is necessary for departure from the ESC state, it may not be sufficient for installation of an alternative GRN, which requires new transcription factor expression and enhancer reconfiguration (Buecker et al., 2014; Factor et al., 2014; Yang et al., 2014). MEK/ERK inhibition is the second component of 2i. ERK1/2 signalling likely contributes directly to naïve GRN destabilisation (Jin et al., 2016; Kim et al., 2014; Yeo et al., 2014), but its role in ESC transition to multi-lineage competence (Kunath et al., 2007; Stavridis et al., 2007) is also anticipated to include transcriptional activation (Tee et al., 2014; Williams et al., 2015).

Here we sought to characterise drivers of the naïve to formative transition that might act in parallel with TCF3, and examine whether genetic deletions might replace 2i and maintain naïve ESC self-renewal.

RESULTS

Identification of ETV5 as a candidate driver of progression from naïve to formative pluripotency

To identify factors that may mediate the effect of MEK/ERK inhibition in driving pluripotency network transition we inspected results from loss-of-function screens. Among transcription factors, we noted that *Etv5* is the most recurrent hit after *Tcf3* in a random mutagenesis screen (Leeb et al., 2014), and is a high confidence candidate from a genome-wide siRNA screen (Yang et al., 2012). ETV5 is a member of the PEA3 sub-family of ETS transcription factors, along with *Etv1* and *Etv4* (Hollenhorst et al., 2011b; Oh et al., 2012). ETV5 and other ETS factors are typically activated by FGF/ERK signalling through transcriptional upregulation and/or protein phosphorylation (Janknecht et al., 1996; Oh et al., 2012; Selvaraj et al., 2015). ETV5 is considered to be functionally redundant with ETV4 and the two factors are co-expressed in multiple tissues in response to FGF (Liu et al., 2003; Mao et al., 2009; Zhang et al., 2009) or GDNF (Lu et al., 2009).

Etv5 transcripts are readily detected in ground state ESC (Fig 1A). In contrast *Etv4* mRNA is not evident in 2i, but is rapidly up-regulated in transitioning cells. Transcripts for both factors are detected in mouse naïve (E4.5) and formative (E5.5) epiblast, with ETV5 being more abundant (Fig 1B). *Etv1* expression is negligible in ESC and the early embryo. We examined in closer detail the behaviour of *Etv5* and *Etv4* in ESC upon transfer from 2i to N2B27. Ribosome profiling indicated potential for a truncated ETV5 protein isoform (Δ N-ETV5) lacking the first 202 amino acids (Ingolia et al., 2011) that include an N-terminal transactivation domain (N-TAD) (Defossez et al., 1997; Laget et al., 1996). Accordingly, we designed alternative RT-qPCR primer pairs. We detected the ETS domain but not the N-TAD encoding sequence in undifferentiated ESC, indicating expression of Δ N-ETV5 only. In contrast, N-TAD-containing transcripts appear within 4h after 2i withdrawal and persist for 48 hours (Fig 1C). Total *Etv5* transcripts increase initially but decline from 24 hours, implying down-regulation of Δ N-ETV5. *Etv4* expression also rises rapidly on removal of 2i, then reduces. We investigated steady state *Etv4/5* expression in ESC maintained with single inhibitors and LIF. Both canonical *Etv5* and *Etv4* mRNA are up-regulated in conditions when ERK signalling is active (Fig 1D).

Inspection of RNA-seq and H3K4me3 ChIP-seq data substantiated activity of an internal *Etv5* promoter in undifferentiated ESC, whereas transcripts originating from the upstream start site are evident after 2i withdrawal (Fig S1A). Immunoblotting identified a smaller Δ N-ETV5 protein (Figs1E, S1B) that decreased from 24hrs of transition. Conversely, the canonical isoform was absent from undifferentiated cells and up-regulated over the transition time course. Single inhibitor withdrawal showed that canonical *Etv5* is expressed in response to MEK/ERK activation. siRNA mediated knock-down confirmed the specificity of immunoblotting (Fig S1B).

ETV5 fulfils distinct roles in ESC self-renewal and transition

Due to the proposed redundancy between *Etv5* and *Etv4*, we first sourced ESC genetically deficient for both genes (E4/5-dKO) (Lu et al., 2009). These cells were derived in serum and LIF and reported to show reduced proliferation (Akagi et al., 2015). This phenotype is exacerbated in 2i/LIF (Fig S1C). However, we found that the mutant cells could be expanded robustly by omitting the MEK inhibitor and culturing in CH/LIF (Fig S1C). We therefore maintained E4/5-dKO cells in CH/LIF, but for consistency with previous studies (Kalkan et al.,

2017; Mulas et al., 2017) cells were exchanged into 2i prior to assay. A short period of 2i culture has no apparent effect on growth rate or viability (Fig S1C). The assay entails withdrawal of 2i for 48h before replating at clonal density in CH/LIF (Fig 1F). Self-renewal capacity is almost entirely extinguished in parental ESC by 48h. In contrast, E4/5-dKO cells still generate numerous undifferentiated colonies (Fig 1G). This phenotype is eliminated upon expression of cDNA encoding either isoform of Etv5 (Fig 1G), both of which also rescue the growth defect during self-renewal (Fig S1C).

To discriminate functions of Etv4 and Etv5, we employed CRISPR/Cas9 to create single and double knock-outs. We used RGd2 ESC that carry the *Rex1::GFPd2* knock-in reporter of naïve status (Kalkan et al., 2017). Etv5-KO ESC also show reduced expansion in 2i or 2i/LIF but proliferate normally in CH/LIF (Fig S1D). These results indicate that Etv5 plays a specific role in consolidating naïve ESC propagation when MEK/ERK signalling is blocked and Etv4 is not expressed. We expanded cells in CH/LIF and transferred into 2i before assay, as above. Etv4 mutants show no significant delay in GFP downregulation (Fig 1H) or extinction of clonogenicity (Fig 1I). In contrast, deletion of *Etv5* results in impaired exit from naïve pluripotency, measured by perdurance of GFP and persistence of clonogenic cells. Normal GFP down-regulation was restored by expressing either Etv5 isoform (Fig S1E). In Etv5 mutants Etv4 is activated later, but to an enhanced level (Fig S1F). However, the Etv5-KO phenotype is not enhanced in E4/5-dKO ESC (Fig 1H), confirming that Etv4 has little relevance for kinetics of transition from 2i.

These results establish that Etv5 supports ESC self-renewal when ERK signalling is inhibited, and facilitates exit from naïve pluripotency when ERK is active. Although canonical Etv5 is specifically up-regulated prior to exit, either isoform can be sufficient for both functions.

Co-deletion of *Etv5* and *Tcf3* retards but does not prohibit exit from naïve pluripotency

Tcf3 is upregulated in Etv5-KO ESC indicating that the phenotypes are independent (Fig S2 A, B). As *Tcf3* is downstream of GSK3 and Etv5 is regulated by ERK1/2, we tested whether combined deletion of both genes might mimic the effect of 2i and be sufficient to sustain ESC self-renewal. We generated *Etv5/Tcf3* single and double mutants in RGd2 ESC and compared GFP profiles after transfer into N2B27 (Fig 1J). In double knockout (ET-dKO) cells, perdurance of GFP was more pronounced. However, at the end of passage 2 ET-dKO cells showed a substantial fraction of GFP low/negative cells, and undifferentiated ESC were not sustained after replating (Fig 1J). Thus TCF3 and ETV5 act combinatorially to drive pluripotency progression, but the absence of both is not sufficient to prevent loss of ESC identity.

Deletion of *Rbpj* delays naïve state exit

We re-inspected the candidate regulators to identify a factor that might complement ETV5 and TCF3 to enforce exit from naïve pluripotency. The repressor RBPJ was detected in a haploid ESC mutagenesis screen (Leeb et al., 2014). RBPJ is expressed in the naïve epiblast in the embryo (Fig S2C). RBPJ is nuclear localized in ESC (Fig 2A) and RBPJ mRNA and protein are up-regulated upon 2i withdrawal, a response that is enhanced in Etv5-KO and *Tcf3*-KO cells (Figs S2D-F). RBPJ is therefore a candidate complementary regulator, regulated by both ERK and GSK3, and acting through uncharacterised targets.

We inactivated *Rbpj* in RGd2 ESC using CRISPR/Cas9. *Rbpj* deficient ES cells adopted a more flattened colony morphology but showed no markers of differentiation (Fig S2G) growth in 2i remained similar to parental ESC (Fig S2H). Upon withdrawal from 2i, *Rbpj*-KO cells exhibit delayed down-regulation of Rex1-GFPd2 and persistence of clonogenic ESC at 42hrs, consistent with siRNA results (Leeb et al., 2014) (Fig 2B, C). Rex1 down-regulation timing is restored upon expression of an *Rbpj* transgene, which also rescues domed colony morphology (Fig S2G, I).

RBPJ is best known for a role in the NOTCH pathway in which activated NOTCH intracellular domain induces a switch from repression to activation of target genes (Kopan and Ilagan, 2009). However, RBPJ can also regulate genes independently of NOTCH (Castel et al., 2013; Johnson and Macdonald, 2011). Absence of most known NOTCH transcriptional targets (Fig S2J), despite detectable expression of NOTCH ligands and pathway components (Fig S2K), suggests that the NOTCH pathway may not be significant during naïve ESC transition. Nonetheless, to test if NOTCH might be relevant to exit dynamics, we employed γ -secretase inhibitors to block production of NOTCH intracellular domain. We did not observe any effect on Rex1-GFP down-regulation (Fig S2L). Thus RBPJ may act purely as a repressor during naïve state exit.

By RNA-seq we found 405 upregulated and 705 downregulated genes in *Rbpj*-KO ESC in 2i ($p_{adj} \leq 0.05$, $FPKM \geq 1$) (Table S1), with functions in multiple processes (Fig S3A, D). Since *Rbpj* deletion did not affect ESC self-renewal (Fig S2H), we focussed on differential expression during transition. At 16h after 2i withdrawal (N16h), 2341 genes were up- and 355 down-regulated (Table S1). KEGG pathway analysis shows enrichment in both 2i and N16h for cell adhesion, focal adhesion and ECM-receptor interactions (Fig S3A, B), reflected in expression of laminins, integrins, collagens and cadherins (Fig S3C). This is in line with observations in *Rbpj* mutant fibroblasts (Castel et al., 2013) and likely explains the morphology of *Rbpj*-KO ESC. Formative pluripotency markers *Lef1*, *Dnmt3b* and *Pou3f1* fail to be up-regulated (Figs 2D, S3E). Reduced expression of FGF/MAPK pathway components and NOTCH receptors is also evident. Among the top upregulated genes are targets of canonical NOTCH signalling known to be repressed by RBPJ, including *Id1*, *Id3*, *Id4* and *Hes1* (Main et al., 2010; Meier-Stiegen et al., 2010) (Figs 2D, S3E, S4A). Repressed targets of *Hes1* (Kobayashi et al., 2009), are among the top downregulated genes, consistent with increased *Hes1* levels. ID factors and HES1 have previously been shown to impede ESC differentiation (Davies et al., 2013; Kobayashi and Kageyama, 2010; Ying et al., 2003; Zhou et al., 2013). *Id3* is highly expressed in *Rbpj* mutants (Fig S4A). CRISPR/Cas9 mediated mutation of *Id3* in RGd2 ESC slightly diminished GFP levels and appeared to accelerate downregulation. However, *Id3* knockout did not restore exit kinetics in *Rbpj*-KO ESC (Fig S4B). We also mutated *Hes1* and found no effect (Fig S4C).

To widen the search for relevant targets we examined pluripotency factor expression in *Rbpj* mutants and noted that *Tbx3* and *Nanog* are among the top 200 up-regulated genes during transition (Figs 2D, F). A previous ChIP-seq study reported these genes amongst candidate RBPJ targets in F9 embryonal carcinoma cells (Lake et al., 2014). We employed ChIP-PCR to examine RBPJ binding at the reported *Nanog* and *Tbx3* sites and confirmed localisation proximal to both genes (Figs 2F, G, S4D). Furthermore, there is a palindromic RBPJ binding motif (Castel et al., 2013) within the binding site at the *Nanog* transcriptional start site (Fig 2F). We used siRNA to knock down *Nanog* and *Tbx3* in *Rbpj* mutants and found in both cases that exit kinetics and clonogenicity are almost fully restored (Fig 2H, I). We also tested the effect of these knockdowns in *Etv5* mutants (Fig S4E). NANOG depletion reverts the *Etv5*-KO exit delay phenotype, reflecting its general importance for sustaining naïve pluripotency, whereas TBX3 siRNA has little or no effect, indicating that its role is specific to the RBPJ context.

These data indicate that up-regulation of RBPJ promotes extinction of naïve pluripotency principally by extinguishing expression of *Nanog* and *Tbx3*. Interestingly, intersection of the ChIP-seq data with transcriptome data from *Rbpj*-KO cells identified 401 potential directly repressed genes (Fig S4F, Table S2), including additional genes associated with ES cell self-renewal notably the LIF signal transducer STAT3. Significantly, however, only 8 of these candidates overlap with high confidence TCF3 repressed targets (Fig S4G, Table S2) (Martello et al., 2012).

Elimination of *Etv5* and *Rbpj* allows self-renewal supported only by GSK3 inhibition

In light of their independent regulation (Fig S2A-F), we created combined mutants for both *Etv5* and *Rbpj* (ER-dKO), and *Rbpj* and *Tcf3* (RT-dKO). Double mutants show a stronger phenotype, but undifferentiated ESC cannot be expanded beyond two passages in N2B27 (Fig 3A). However, deficiency for *Etv5* and *Rbpj* is sufficient to sustain self-renewal in GSK3 inhibitor (CH) only. Parental ESC cannot be propagated in these conditions beyond passage 2 (Fig 3B). Both single mutants can also be maintained in CH (Fig S4H), but expand slowly (Fig S4I), whereas ER-dKO cells proliferate similarly to parental RGd2 cells in 2i (Fig S4I) and remain uniformly GFP-positive (Fig 3B). After 5 passages in CH, we assayed colony formation in 2i/LIF as a measure of naïve ESC frequency. ER-dKO cells generated undifferentiated colonies with undiminished efficiency relative to RGd2 cells maintained in 2i (Fig 3C,D-a,b). Furthermore, they could also form colonies robustly in CH (Fig S4J), a property that is lost in parental ESC after 2 passages. These results demonstrate a combined effect of *Etv5* and *Rbpj* deletion that enables self-renewal without MEK inhibition or LIF.

Triple deletion of *Etv5*, *Rbpj* and *Tcf3* renders ESC self-renewal constitutive

We then generated triple knockouts for *Etv5*, *Rbpj* and *Tcf3* (ETR-tKO). Like *Rbpj*, *Tcf3* and ER-dKO mutants, ETR-tKO cells were flattened but undifferentiated and uniformly GFP positive in CH/LIF (Figs 3E, F, S5A). In contrast to other mutants, two independently generated ETR-KO clones maintained robust GFP expression in N2B27 (Fig 3G), expanding constantly although more slowly than in CH/LIF (Fig S5B, C). After 10 passages in N2B27, only a minor GFP-negative population emerged (Fig 3G). ETR-tKO cells passaged in N2B27 generated numerous alkaline phosphatase positive colonies on replating at clonal density in 2i/L and also in N2B27 only (Fig 3C,D-c,d). Immunostaining of ER-dKO cells in CH and ETR-tKO cells in N2B27 showed relatively homogeneous staining for OCT4 and for naïve pluripotency factors NANOG and TFCP2L1 (Fig 3H). Under neural differentiation conditions, ETR-tKO cells maintained Nanog and Klf4 protein expression with no induction of neural markers SOX1 or TuJ1 (Fig 3I).

We examined whether conversion to primed EpiSC (Brons et al., 2007; Guo et al., 2009; Tesar et al., 2007) is impeded in the various mutants. Cells were transferred to medium containing Activin, Fgf2 and Wnt pathway inhibitor XAV939 (Sumi et al., 2013), hereafter AFX. Over 3 passages RGd2 lines converted into epithelial EpiSC with complete loss of GFP but retention of alkaline phosphatase (Fig 4A-C). *Tcf3*-KO and *Rbpj*-KO mutants similarly converted efficiently to EpiSC. In contrast, *Etv5*-KO cells down-regulated GFP (Fig 4B), but displayed distinct morphology by passage 2 (Fig 4A-c, d). By passage 3 cultures differentiated into fibroblast-like cells (Fig 4A-e, f) and contained only occasional patches of alkaline phosphatase positive cells (Fig 4C). Only one culture eventually yielded with EpiSC-like cells and these cells deviated from the EpiSC state, with lower expression of *Pou3f1* and almost no *Fgf5* or *Otx2* (Fig 4D). In contrast to all the above, ETR-tKO mutants retained a significant proportion of GFP cells, even after 10 passages in AFX (Fig 4E). They maintained substantial expression of naïve markers *Klf2* and *Tfcp2l1* with low expression of *Otx2*, *Pou3f1* and *Fgf5* (Fig 4D).

We examined embryoid body formation in serum, an inductive system for multilineage differentiation (Doetschman et al., 1985). Single mutants down-regulated naïve markers but failed to up-regulate the mesoderm marker Pax3 or neural marker Pax6 (Fig 4F). Endoderm markers were less affected, but this may reflect extraembryonic differentiation without formative transition (Smith, 2017). In ETR-tKO cells upregulation of formative and lineage markers was severely diminished or delayed while expression of naïve pluripotency markers persisted.

We investigated whether paracrine signalling might contribute to ETR-KO cell resistance to transition. We labelled ETR-tKO cells with the monomeric Kusabira Orange (mKO) reporter and set up mixed cultures with a minority (5%) of RGd2 test cells. GFP downregulation kinetics were unaltered (Fig S5D), demonstrating that the ETR-tKO phenotype is cell intrinsic.

Finally, we introduced a transgene for re-expression *Etv5* and *Tcf3* in ETR-tKO mutants. Doxycycline induced expression was lower than endogenous wild type levels, but cells initiated downregulation of *Rex1* and *Nanog*, and upregulation of *Pou3f1* (Fig S5E, F). After 3 days in N2B27 alone, NANOG protein was absent from a high proportion of Dox-treated cells while it remained uniformly expressed in untreated cells (Fig S5G). This rescue experiment indicates that the transition delay phenotype is reversible and directly attributable to the mutated genes.

Whole transcriptome analysis of *Etv5/Rbpj* and *Etv5/Tcf3/Rbpj* mutant ESC

We performed RNA-seq (Table S3) and compared mutant cells with RGd2 cultures in 2i, N2B27 for 16h or 72h (N16h or N72h), or CH for 2 passages (CHp2). At 16h ESC are poised for transition but will regenerate ESC colonies at high efficiency if restored to 2i/LIF (Kalkan et al., 2017). Hierarchical clustering (Fig 5A) divides N72h and CHp2 from other samples, consistent with having exited the ESC state. ETR-tKO cells cultured in N2B27 and ER-dKO cells in CH form a sub-cluster between 2i and N16h samples. Principal component analysis (PCA) discriminates on PC1 samples before and after exit (Fig 5B), while PC2 separates 2i from transitional cells. ETR-tKO cells are close to 2i samples but displaced towards N16h.

Pluripotency factor profile is similar between ETR-tKO and naïve ESC, with some modulation in levels (Fig 5C). Expression of some formative markers is detectable, but at levels below those in N16h cells. Lineage markers are absent or very lowly expressed (Fig 5D; Tables S2, S3). As in *Rbpj* single mutants, NOTCH targets, focal adhesion and ECM genes are upregulated, along with actin cytoskeleton components (Fig 5E, F). MEK/ERK pathway components and targets are upregulated while expression of Wnt target genes is reduced, in line with absence of 2i (Fig 5E, F). Several metabolism and lysosome related genes are downregulated, which may relate to slower growth of ETR-tKO cells (Fig S5F).

These results establish that the naïve pluripotency factor network is intact and the transition to formative pluripotency is barely initiated, both for *Etv5/Rbpj* mutants cultured in CH and for triple mutants in N2B27 only.

Triple knock out cells colonise chimaeras but do not convert to post-implantation epiblast

We then examined whether ETR-tKO cells retain functional proximity to naïve epiblast. We introduced a constitutive H2B-tdTomato reporter and performed injections into 8 cell embryos that were then cultured to the expanded blastocyst stage. In 9/9 blastocysts, mutant cells extensively colonised the epiblast, outnumbering the host cells similarly to parental RGd2 cells (Fig 6A). Contribution was confined to the Sox2 positive epiblast, with no cells detectable in primitive endoderm or trophoblast. Thus ETR-tKO cells retain the ability of undifferentiated ESC to survive, proliferate, and colonise the epiblast exclusively (Alexandrova et al., 2016).

We then examined behaviour of ETR-tKO cells in post-implantation development following uterine transfer of injected embryos. Mutant cells were present in 20/20 embryos recovered at E6.5 and E7.5. Unlike RGd2 chimaeras, which showed distribution of ESC progeny throughout the egg cylinder epiblast, most of the embryos injected with mutant cells had abnormal or rudimentary egg cylinders (see Table S4 for phenotypes and numbers). Mutant cells did not intermingle with host cells (Fig 6B-D). They retained expression of *Rex1*-GFPd2 and of *Nanog*,

and failed to up-regulate *T (Brachyury)* or *Pou3f1*. In some cases ETV5-KO contributions were large and extended beyond the embryonic/extraembryonic boundary (Table S4).

We conclude that triple mutant cells are unable to adopt identity of post-implantation epiblast and consequently cannot respond to inductive signals for germ layer specification. Persistence in a naïve-like state disrupts development of the host epiblast.

ETV5 regulates network components of both naïve and formative pluripotency

To illuminate how ETV5 regulates both self-renewal and transition we performed RNA-seq and ChIP-seq in 2i and N16h. We identified 754 ChIP-seq peaks in 2i and 1020 at N16h, with only 392 in common (Table S5, Fig 7A). Thus there is a major change in ETV5 genome location early in the ESC transition process. As observed in other cell types for ETV5 and ETS factors in general (Hollenhorst et al., 2007; Zhang et al., 2017), ETV5 peaks were enriched at promoters (Fig S6A). RNA-seq in 2i revealed 77 down-regulated genes ($FC \leq 0.67$) and only eight upregulated genes ($FC \geq 1.5$) associated with ETV5 peaks (Fig S6B, Table S6)), consistent with function of ΔN -ETV5 as a transcriptional activator. Targets include genes with potential roles in proliferation and maintenance of ESC (Fig S6B): *Sal1* (Novo et al., 2016); *E2f2* (Wang and Baker, 2015); *Id3* (Ying et al., 2008); and most notably *Klf5*, which supports robust ESC proliferation (Ema et al., 2008). Activation of these genes may explain the contribution of ETV5 to ESC expansion. On the other hand, ETV5 binding is also detected at genes encoding transcription factors and epigenetic regulators associated with ESC transition or recovered in exit screens (Fig S6B). By priming transcription of these genes, ETV5 may prepare naïve cells for rapid progression. Curiously, *Otx2* was upregulated in *Etv5*-KO cells in 2i, although this was not sustained during transition (Figs S6B, S7A).

At N16h we found ETV5 binding proximal to 163 ($FC \geq 1.5$) of 3672 up-regulated genes ($p_{adj} \leq 0.05$, $FPKM \geq 1$) (Fig S6C, Table S6). These include transcription factors, components of H3K4 methyltransferase complex, negative regulators of Ras/ ERK pathway, and Tgf- β pathway members. *Id3* and some naïve transcription factors, are also represented. These data present the possibility that ETV5 might participate in transcriptional repression by an unknown mechanism and thereby contribute to shutting down the naïve GRN. Intersection with TCF3 and candidate RBPJ repressed targets showed limited overlap (Fig S6D).

Among 346 downregulated genes at 16h, 16 are associated with proximal ETV5 binding (Fig 7B). These include genes in metabolic pathways and involved in calcium signalling. This is of note because metabolic resetting is an early feature of ESC transition (Fiorenzano et al., 2016; Kalkan et al., 2017; Zhou et al., 2012). Prominent formative pluripotency markers *Lef1*, *Fgf5* and *Pou3f1* are also represented. ETV5 binds to enhancers associated with these three genes (Fig 7C-E). The enhancers are activated in EpiLC (Buecker et al., 2014), a transient population obtained by plating ESC in Activin/Fgf2/KSR for 48h (Hayashi et al., 2011). We found that ETV5 remains bound in EpiLC (Fig S7B). *Etv5* mutants show impaired *Rex1* downregulation (Fig 7F) and reduced expression of the three genes in EpiLC culture (Fig 7G). Furthermore, gain of H3K27Ac at the enhancers is diminished in mutants (Fig 7H), suggesting that ETV5 may promote H3K27 acetylation.

We used CRISPR/Cas9 to mutate *Pou3f1* and *Lef1* (Fig S7C). However, in neither single nor double mutants did we observe a delay in exit (Fig S7D). The marker profile of *Lef1/Pou3f1* double mutants at 48h was also similar to parental RGd2 cells, although a modest reduction in *Sox2*, *Sox3* and *Fgf5* was apparent (Fig S7E). We also noted that ETV5 binds to the *Oct4* proximal enhancer that is required for expression in post-implantation epiblast (Fig S7F) (Yeom et al., 1996), and found that *Oct4* expression was maintained at ES cell levels during mutant cell conversion to EpiLC (Fig S7G). We then examined potential wider-reaching actions of ETV5. We partitioned non-promoter ETV5 peaks, which include enhancers, into 3 groups: 2i

only; N16h only; shared. Across these regions we computed levels of chromatin marks associated with active enhancers; H3K27Ac, H3K4me1 and p300, from (Buecker et al., 2014)(Fig 7I). We found that 2i-specific ETV5-bound regions lose H3K27Ac and p300 upon conversion to EpiLC, whereas across 208 loci that gain ETV5 at 16h, there is a marked increase in H3K27Ac and p300 in EpiLC. A more modest gain in H3K27Ac is apparent across shared regions. Thus ETV5 relocates from naïve pluripotency-specific enhancers to formative/EpiLC enhancers upon 2i withdrawal. In contrast, promoters associated with ETV5 are largely devoid of p300 and show loss of H3K27Ac in EpiLC (Fig S7H). ETV5 binding is coincident with p300 at enhancers (Figs 7C-E, I). This is of note because p300 has been shown to acetylate ETV1 and ETV4, increasing transactivation potential (Goel and Janknecht, 2003; Guo et al., 2011a), a mechanism likely also to operate for ETV5. Depletion of p300 has an ESC transition delay phenotype (Leeb et al., 2014), consistent with p300 and ETV5 cooperating to commission formative enhancers.

DISCUSSION

This study demonstrates that elimination of two transcriptional repressors and one activator effectively prohibits mouse ESC progression to lineage competence. TCF3, RBPJ and ETV5 serve complementary and partially overlapping functions in driving exit from the ESC ground state and initiation of formative pluripotency. Cells lacking all three factors are trapped in an ESC-like condition from which they can only rarely escape, even in the presence of strong differentiation stimuli.

Absence of TCF3 permits ESC propagation in MEK inhibitor alone (Wray et al., 2011), while combined deletion of ETV5 and RBPJ sustains self-renewal with GSK3 inhibition only. Triple knock out cells are liberated from requirement for both inhibitors and exhibit constitutive self-renewal. These cells are stalled at a very early stage in transition. They retain uniform expression of naïve pluripotency factors and high clonogenicity. Robust colonisation of the naïve epiblast demonstrates they remain functionally within the ESC compartment. However, ETV5-tKO cells cannot advance from this state even in the powerful inductive environment of the post-implantation embryo. These observations are consistent with evidence that ES cells may self-renewal autonomously if differentiation is prevented.

TCF3 is well characterised as an ESC regulator (Cole et al., 2008; Guo et al., 2011b; Pereira et al., 2006; Wray et al., 2011). RBPJ by contrast has not previously been studied in this context. RBPJ is known to repress *Hes1* and *Id* genes, factors that can delay or reverse pluripotency progression in ESC cultured in serum or BMP (Davies et al., 2013; Ying et al., 2003). In the peri-implantation embryo, RBPJ may counterbalance BMP to curtail persistence of naïve epiblast. However, during defined in vitro transition the critical contribution of up-regulated RBPJ is to extinguish expression of naïve factors, in particular *Nanog* and *Tbx3*, and prevent re-ignition of the naïve GRN. Elimination of *Tbx3* may also be important to restrict potency for extraembryonic endoderm (Lu et al., 2011).

Combined deletion of *Etv4* and *Etv5* compromises proliferation and differentiation of ESC (Akagi et al., 2015). We find that both phenotypes are primarily attributable to *Etv5*. This is in line with post-implantation lethality by E8.5 of ETV5 ETS domain deletion (Lu et al., 2009), while *Etv4*-null mice are viable (Arber et al., 2000; Laing et al., 2000). *Etv5* mutant mice that carry an N-terminal deletion are also viable (Chen et al., 2005), supporting functionality of Δ N-ETV5. Δ N-ETV5 may have altered signal sensitivity and partner interactions, which may be of relevance in cancers in which PEA3 family members are frequently mis-expressed (de Launoit et al., 2006; Hollenhorst et al., 2011a). Δ N-ETV5 supports ESC propagation when ERK signalling is inhibited, potentially via direct regulation of *Klf5*. When ERK is active in self-

renewal conditions, ETV5 is dispensable, likely due to activation of an alternative ETS factor such as ETV4 or GABPA.

During naïve GRN collapse, ETV5 pivots from supporting naïve ESC propagation to activating the formative pluripotency programme. Absence of ETV5 derails installation of the formative GRN and also impedes exit from naïve pluripotency. This phenotype is distinct from *Tcf3* and *Rbpj* mutants, in which there is a delay in exit but no major compromise in subsequent transition or formation of EpiSC. However, mutations in peri-implantation epiblast factors implicated in formative pluripotency, such as *Pou3f1* and *Lef1*, do not substantially delay naïve state exit. Initial handover to the formative GRN may therefore be specifically dependent on ETV5. We surmise that in the absence of ETV5, the network switch is not initiated and cells transiently retain, or revert to, naïve status. Under influence of TCF3 and RBPJ they eventually exit, but then mostly succumb to miscellaneous differentiation or death, although there is some rescue by ETV4 or other pathways.

PEA3 factors are known as transcriptional activators (Oh et al., 2012). Interestingly, even in ground state ESC, Δ N-ETV5 may prime transcription of some early transition genes (Fig S6B). Upon 2i withdrawal, ETV5 occupies new genome locations, many associated with enhancers that become active during or after transition. ETV5 is phosphorylated by active ERK1/2 and is a probable target for acetylation by p300. These effects likely lead to the observed relocation whereby ETV5 can rapidly contribute to commissioning the formative GRN.

ETR-tKO cells retain core features of ESC identity but operationally appear nullipotent because they are unable to execute the formative transition. We conclude that timely and correct developmental progression from naïve pluripotency is determined by three functions: TCF3 triggers dissolution of the naïve GRN; RBPJ enforces exit by preventing reversion; ETV5 commissions the successor formative GRN. Additional regulators contribute (Betschinger et al., 2013; Kalkan and Smith, 2014; Leeb et al., 2014; Li et al., 2018), but these three effects may be the major drivers. Speed and efficiency depend on temporal coordination and it is striking that all three components are already present in naïve ESC, although levels and activity change during transition. It will be of interest to compare with mammals that have prolonged pluripotency progression, including primates (Nakamura et al., 2016; Smith, 2017). Future research will also reveal whether dissolution, enforcement, and initiation actions may commonly be combined to provide a triple lock for secure cell state transition.

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Author Contributions

Conceptualization, T.K. and A.S.; Methodology, T.K.; Formal Analysis, T.K., S.B., E.D., M.R. and P.L.; Investigation, T.K., C.M., T.L., S.M. and J.N.; Writing, T.K. and A.S.; Supervision, J.N. and A.S.

Declaration of Interests

The authors declare no competing interests.

Main Figure Legends

Figure 1. *Etv4/5* expression and function

(A,B) RNA-seq expression values (FPKM or RPKM) for naïve and transitioning ESC, and early embryo lineages. Error bars represent standard deviation (SD) from (A) 2 and (B) 3 independent biological replicates.

(C,D) RT-qPCR expression in 2i and after 2i withdrawal, and in 2i/L components for 3 passages. Data are means \pm SD from: (C) 2 wells of cells differentiated in parallel; (D) 3 independent biological replicates. L=LIF, CH= CHIR99021, P= PD0325901.

(E) Western blot with anti-Flag antibody on *Etv5*-3XFLAG knock-in ESC.

(F) Schematic for panels G-I.

(G) Quantitation of colony assays on WT (wild-type), *Etv4/5*-dKO ESC, and *Etv4/5*-dKO ESC expressing monomeric Kusabira Orange (mKO), Δ N-*Etv5* or canonical *Etv5* transgenes. Error bars show SD from 2 technical replicates.

(H) Rex1-GFP profiles of RGd2 and independently generated clonal lines (c) of *Etv4*-KO; *Etv5*-KO and *Etv4/5*-dKO at 25 and 42 hours post-2i withdrawal. RGd2-2 is a clonal line derived from parental RGd2-1.

(I) Colony assay.

(J) GFP profiles for parental RGd2 and mutant ESC lines at 72h post-CH/LIF withdrawal (N72h) and end of passage 2 (p2). Red cross indicates failure of replating after passage. ET= *Etv5*/Tcf3.

See also Figures S1 and S2.

Figure 2. *Rbpj* expression and function

(A) *Rbpj* Western blot: T= total cell lysate, C= cytoplasmic fraction, N= nuclear fraction. Oct4 and GAPDH were used as loading controls for nuclear and cytoplasmic fractions, respectively.

(B) GFP profiles of RGd2 and three clonal *Rbpj* mutant lines in N2B27 at 25h (N25h) and 42h (N42h) post-2i withdrawal.

(C) Colony assay.

(D) MA-plot showing mean expression against fold change per gene in *Rbpj*-KO ESCs at 16h post-2i withdrawal (N16h). Gene symbols and coloured tags are shown for selected genes listed.

(E) RNA-seq expression values for naïve pluripotency factors in RGd2 and *Rbpj*-KO ESC in 2i and at N16h. Error bars show SD from biological replicates plated in parallel; 3 independent clonal lines for *Rbpj*-KO, 2 different lines for RGd2 (one parental and one clonal)

(F) UCSC genome browser tracks for *Nanog* and *Tbx3* loci showing normalized RNA-seq read coverage for parental and *Rbpj*-KO ESC at N16h. RBPJ binding sites are indicated with red arrowheads. The RBPJ-binding motif within the *Nanog* locus is highlighted.

(G) ChIP-qPCR for binding sites shown in Fig 2F and Fig S4E. Two primer sets were used for the *Tbx3* locus. Y axis shows absolute enrichment normalized to input DNA for each sample. Error bars indicate SD from two ChIP replicates.

(H) GFP profiles at 40h after 2i withdrawal following a 7-hour period of siRNA transfection.

(I) Colony assay at 40h after 2i withdrawal.

See also Figures S2-4 and Tables S1, S2.

Figure 3. Dual and triple knock out phenotypes

(A) GFP profiles of RbpJ-KO (R-KO), Etv5/Rbpj-dKO (ER-dKO), Rbpj/ Tcf3-dKO (RT-dKO) ESC at 72h post-CH/LIF withdrawal (N72h) or at the end of passage 2 (p2). Red crosses indicate failure of replating upon passage.

(B) Profiles of RGd2 and ER-dKO ESCs cultured in CHIRON (CH) only.

(C, D) Clonogenicity in 2i/LIF or N2B27. Error bars show SD from 2 technical replicates.

(E) GFP Profiles of ETR-tKO and RGd2 ESCs cultured in CH/LIF.

(F) Phase contrast images. Scale bar= 75µM.

(G) GFP Profiles of ETR-tKO ESCs cultured in N2B27 only

(H) Immunofluorescent staining (IF) of RGd2 ESCs cultured in 2i, ER-dKO in CH and ETR-tKO in N2B27 after 6 passages.

(I) IF after 8 days of neural differentiation.

Images were taken using 20x (H) and 10x (I) objective.

See also Figures S4 and S5.

Figure 4. Transition failure of Etv5 and triple knock out ESC

(A) Phase contrast images of RGd2 and Etv5-KO ESCs during first three passages (p1-3) in AFX taken using a 10x objective. Scale bar= 75µM.

(B) GFP profiles at the end of p1

(C) Alkaline phosphatase staining at the end of p3

(D, E) RT-qPCR and GFP profiles after 10 passages in AFX

(F) RT-qPCR on embryoid bodies on days 3-9. Day 0 is starting ESC in CH/LIF. Error bars in D and F show SD from 2 technical replicates for qPCR .

Figure 5. Transcriptome analysis of single and combined mutants

(A, B) Hierarchical clustering and PCA plot based on normalized gene expression for all genes. * denotes cells expanded in CH/LIF and switched to 2i for 48hrs prior to sample conditions.

(C, D) Heatmaps showing relative expression for: pluripotency genes; lineage markers. Values are shown as Log2 fold change of RNA-seq read counts relative to RGd2 2i-p5. Only the genes with a mean expression value of FPKM \geq 1 in either RGd2 2i-p5 or ETR-tKO N-p5 samples were included. Genes were sorted by mean expression within each group.

(E) KEGG pathway enrichment for differentially expressed genes.

(F) MA-plot showing mean expression against fold change per gene in ETR-KO cells cultured in N2B27 for 5 passages (ETR-tKO N-p5) vs RGd2 2i-p5 sample. Gene symbols are shown for selected genes listed below.

See also Tables S2, S3.

Figure 6. Chimaera contribution and perturbation by triple knock out ESC

Reporter fluorescence and whole mount immunofluorescence staining on chimeric embryos obtained from RGd2 or ETR-tKO ESCs labelled with H2B-tdTomato (red).

(A) In vitro matured blastocysts at E4.5 stained for Gata4 and Sox2. Scale bar=50µm.

(B) E6.5 embryos with T (Brachyury) staining and Rex1-GFP fluorescence. Arrowheads in the lower DAPI panel point to separate ETR-tKO chimeras. Scale bar=100µm.

(C) E7.5 embryos with Pou3f1 (Oct6) and Nanog staining. Scale bar=200µm

(D) E7.5 T and Nanog staining. Scale bar=200µm.

Note the different magnifications for RGd2 and ETR-tKO chimeras in C and D.

See also Table S4

Figure 7. Etv5 association with transcriptionally dynamic genes

(A) Numbers of ETV5 ChIP-seq peaks in 2i or at 16h post-2i withdrawal (N16h).

(B) Heatmap showing relative expression of downregulated genes in *Etv5* mutants (fold ≤ 0.66) at 16h post-2i withdrawal (N16h) with associated Etv5 binding.

(C-E) UCSC Genome browser tracks showing normalized ChIP-seq read coverage for Etv5 and H3K4me3 (this study), p300, H3K27Ac and H3K4me1 (Buecker *et al* , 2014).

(F) GFP profiles of EpiLCs (48h in Activin/Fgf2/KSR) generated from RGd2 ESCs or ETV5-KO ESCs (2 clonal lines).

(G) RT-qPCR on time course samples during EpiLC formation. Error bars show SD from 2 wells of cells differentiated in parallel.

(H) ChIP-qPCR showing H3K27Ac levels on upstream and downstream loci adjacent to the Etv5 peaks shown in C-E. Y-axis shows absolute enrichment normalized to input DNA from each sample. ChIP was performed in duplicate (1 and 2) for each sample. Error bars show SD from 2 qPCR replicates.

(I) Mean read coverage for p300, H3K27Ac and H3Kme1 (ChIP-seq from Buecker *et al*, 2014) on “non-promoter”-associated ETV5-bound loci. Read depth scaled to 1x.

See also Figures S6, S7 and Tables S5, S6.

STAR Methods

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact Austin Smith, austin.smith@cscr.cam.ac.uk

Experimental Model and Subject Details

Mice used in these studies were adult females aged 6-10 weeks. The CD1 strain was used to provide embryos and CBA/BL6 F1 animals were employed as transfer recipients. Animals in the facility tested positive for *Helicobacter* but negative for other specific pathogens. Studies were performed in a UK Home Office designated facility in accordance with EU guidelines for the care and use of laboratory animals, and under authority of a UK Home Office project licence. Use of animals in this project was approved by the Animal Welfare and Ethical Review Body for the University of Cambridge.

ES cell culture

Since Etv5-KO ESCs cannot be cultured long-term in the presence of PD, for consistency across experiments presented in this paper and with previous studies (Kalkan et al, 2017, Mulas et al, 2017), all cell lines were routinely cultured in CH/LIF then exchanged to 2i or 2iLIF for a total of 48hrs before the assay. For CH/LIF cultures, ES cells were plated at 1×10^4 cells cm^{-2} in CH/LIF on plates coated with 0.1% Gelatine (Sigma-Aldrich, G1890). Media was refreshed every other day and cells were passaged every 3 days. For passaging, cells were dissociated with Accutase (Millipore, SCR005) for 5 mins, 5-10x volume of wash buffer [DMEM/F12, 0.03% BSA Fraction V (Thermofisher)] was added, cells were spun and resuspended in fresh CH/LIF. Culture media used in the experiments consisted of N2B27 (made in house) supplemented with CH/LIF, 2i (CH/PD) or 2i/LIF at the following final concentrations: PD0325901 (PD), 1 μM ; CHIR99021 (CH), 3 μM ; LIF (prepared in house), 1:1000. To calculate growth rates, cell lines were plated at equal density, and counted at the end of each passage using a Vi-CELL Automated Cell Viability Analyzer (Beckman-Coulter).

Monolayer differentiation, clonogenicity assays and flow cytometry

For analyses of kinetics of exit from naïve pluripotency, CH/LIF cultures were exchanged to 2i for 24hrs, dissociated with Accutase, plated in 2i at 1.5×10^4 cells cm^{-2} and cultured for 24hrs prior to withdrawal of inhibitors. For flow cytometry, ESCs were dissociated using Accutase and diluted 1:5 in FACS buffer [PBS, 5% FBS]. ToPro-3 (Invitrogen) was added at a concentration of 0.05 nM to label membrane-compromised cells. Flow cytometry was performed on a Dako Cytomation CyAn ADP high-performance cytometer, using the same voltage settings for all experiments and results were analyzed with Flowjo. Representative GFP profiles from at least 2 independent experiments are shown throughout the paper. For clonogenicity assays, cells were dissociated at 40-48h post-2i withdrawal and following resuspension in appropriate media, cells were plated at single cell density (~500 cells/ 6-well) in 2i/LIF or CH/LIF on plates coated with 1% Laminin (Sigma, Cat. L2020) in duplicate. At day 6 alkaline phosphatase staining was performed using AP Kit (Sigma-Aldrich). Plates were scanned using Cell Selector (Aviso) and colonies were scored manually.

Notch Inhibition

RGd2 ES cells were treated with γ -secretase inhibitors DBZ (CAS 209984-56-5, Calbiochem) or DAPT (565770, Merck) at the concentrations shown in figure legends. DMSO was used as vehicle control.

Neural differentiation

Plates were coated with Laminin (~10µg/ml in PBS) for at least 4 hours to overnight at 37°C. Laminin was aspirated and ESCs were plated directly in N2B27 onto laminin-coated plates at a density of 1.0×10^4 cells/cm². N2B27 was refreshed on day 2 and every day thereafter.

ES cell to EpiSC conversion and EpiSC culture

ES cells were plated in N2B27 supplemented with Activin A (20ng/ml), Fgf2 (12.5ng/ml) and XAV939 (1µM) on Fibronectin-coated (Millipore, FC010) plates at a density of 1×10^4 cells/cm². Media was refreshed on the 2nd day and cells were passaged every 3 days using Accutase (Millipore, SF006). To enhance plating efficiency ROCK-inhibitor Y27632 (1mM) was included for the first 6-12 hours following plating and then removed.

Differentiation of ES cells in embryoid bodies (EB)

Single EBs per well were generated by sorting 1500 ES cells using a MoFlo Flow Sorter (Beckman Coulter) into a well of PrimeSurface96U plates (Sumitomo Bakelite) containing GMEM supplemented with GMEM, L-Glutamine (2mM), NEAA, Sodium Pyruvate (1mM), non-essential amino acids, β-Mercaptoethanol (100mM) (Life Technologies) and 15% FCS (Hyclone). Twelve EBs were pooled for assay.

Generation of Knock-Out ES cell lines using CRISPR-Cas9-mediated mutagenesis

gRNAs were cloned into PX459 (hSpCas9-2A-Puro) vector. Two gRNAs targeting different exons of a gene were co-transfected into Rex1-GFPd2 ESCs (Kalkan et al, 2017) using FuGENE HD (Promega). Transfected cells were selected using Puromycin (0.5-1µg/ml) between 14h- 86h post-transfection. Clones were picked on day 9, expanded in CH/LIF and assayed by qRT-PCR to detect the genomic deletion. Details of gRNAs and qPCR primers are included in Table S7.

Genetic rescue of Etv5-KO, Rbpj-KO, ETR-tKO ES cell lines

cDNAs encoding *Tcf3*, *Rbpj* and long and short forms of *Etv5* were amplified from total cDNA of RGd2-N16h samples. *Rbpj* and *Etv5* isoforms were cloned into a PiggyBac vector containing a PGK-Hygromycin selection cassette and a CAG promoter to drive constitutive transgene expression. To generate stable “rescue” ES cell lines, 1×10^6 Knock-Out ES cells were co-transfected with PiggyBac constructs (20ng) and PiggyBac transposase (400ng) using 1.5 µl FuGENE HD (Promega) for 14h in CH/LIF in one well of a 6-well dish. From 24h post-transfection cells were cultured in Ch/LIF with Hygromycin (160µg/ml). For inducible expression of *Etv5* and *Tcf3* in ETR-tKO ESCs, an *Etv5*-p2A-*Tcf3* transgene was generated by PCR and cloned into a PiggyBac vector containing a PGK-Hygromycin selection cassette and a TRE3G promoter to drive Doxycycline-inducible transgene expression. 3×10^5 ETR-tKO ES cells were co-transfected with this expression construct (50ng), a CAG-Tet3G-IRES-zeocin construct (50ng) and PiggyBac transposase (100ng), using 1µl FuGENE HD (Promega) for 14h in Ch/LIF in one well of a 6-well dish. From 24h post-transfection cells were cultured with 20µg/ml Zeocin and 160µg/ml Hygromycin. Experiments were performed after at least 10 days of selection of stable transfectants with antibiotics. Doxycycline (100ng/ml) was added to culture media to induce transgene expression.

Generation of mKO- and H2B-TdTomato-labelled ESCs

1×10^6 RGd2 and ETR-tKO ESCs were transfected as above with a Piggybac construct carrying CAG-driven monomeric Kusabira Orange (mKO) (20ng) and a PGK-Hygromycin selection cassette together with PiggyBac Transposase (400ng) and stable transfectants were selected with Hygromycin (160 ng/ml). For TdTomato labelling, 1×10^6 ESCs were transfected with CAG-driven H2B-TdTomato-IRES-Puro construct using 10µl FuGene in one well of 6-well plate and selected with Puromycin (2µg/ml).

siRNA transfection

RGd2 and *Rbpj*-KO ESCs cultured in CH/LIF were switched to 2i for 24hrs before siRNA transfection. Cells were dissociated and resuspended in 2i. 6×10^4 cells were mixed with 700 μ l 2i, 1 μ l Lipofectamine RNAiMax (ThermoFisher) and 2 independent siRNAs for Tbx3 or Nanog or both, at a final concentration of 1.25nM each, and plated in a well of a 24-well tissue culture plate. AllStars negative control siRNA (Qiagen) was used as control. After 7hrs, medium was replaced with N2B27 to initiate differentiation. Please see Table S7 for siRNA catalog numbers.

Immunofluorescence (IF) staining of ES cells

Cells were fixed for 10 min with 4%PFA at RT, followed by permeabilization and blocking in blocking buffer [PBS, 0.1% TritonX-100, 3% donkey serum] for 2hrs at RT. Cells were incubated with primary antibodies in blocking solution overnight at 4 °C, using dilutions shown in Table S7. Alexa Fluor-conjugated donkey secondary antibodies (Molecular Probes) were used at 1:1000 dilution and were incubated with cells for 1hr at RT. Cells were washed with PBS/0.1% TritonX-100 three times for 5mins after primary and secondary antibody incubations. Images were obtained using a Leica 4000B standard fluorescent microscope using a 10x or 20x objective as indicated in the figure legends.

RNA extraction, cDNA synthesis and qPCR

Total RNA was isolated using Relia Prep RNA Miniprep System (Promega). cDNA was synthesized using GoScript Reverse Transcriptase System (Promega) and oligo-dT primers. qRT-PCR was performed with TaqMan Gene Expression (Thermo Scientific) or Universal Probe Library (Roche) assays. Expression levels were normalized to GAPDH for all analyses, except for Figure 1C for which TBP was used. Please see Table S7 for details.

Western blot and sub-cellular fractionation of total cell lysate

To obtain total cell lysate cells were lysed in 1xPBS supplemented with 1%TritonX-100, 0.1%SDS, protease and protein inhibitors (Roche) and sonicated briefly in the Bioruptor (Diagenode) to shear the gDNA. Extraction of nuclear and cytoplasmic fractions was performed with NEPER Nuclear and Cytoplasm Extraction Reagents (ThermoFisher Scientific). Blots were blocked with blocking solution (1xPBS, 1%TritonX-100, 5% skimmed milk) for 2hrs at RT, followed by incubation with primary antibodies for 2hrs at RT or overnight at 4 °C. Primary antibodies and dilutions are listed in Table S6. For detection IRDye secondary antibodies (Licor) were used at 1:2000- 1:5000 dilution and signal intensities were quantified by Odessey (Licor). Antibodies and primer/probes sets are listed in Table S6.

Chromatin immunoprecipitation (ChIP) and ChIP-seq library preparation

ESCs were dissociated with Accutase, washed with culture medium (10x volume) and resuspended in fresh culture medium at 5×10^6 cells per ml. To cross-link chromatin, for 1ml of cell suspension 100 μ l of Fix Solution [0.1M NaCl, 1mM EDTA, 0.5mM EGTA, 50mM HEPES pH 7.5, 11% Formaldehyde] was added, and cells were rotated for 10min at RT. To neutralize the formaldehyde, 157 μ l 1M Glycine was added, and cells were rotated for 5min at RT, followed by spin at 1600g for 5min. Cells were then washed with 1ml ice-cold PBS/BSA 0.03% and spun, repeating 3 times. Protease /Phosphatase inhibitors (Roche) were added in the last wash. Cells were either frozen at -80°C at this point or processed immediately. To obtain nuclear lysates, pellets from 5×10^6 fixed cells were resuspended in ice-cold 1ml buffer LB1 [50mM HEPES pH 7.5, 140mM NaCl, 1mM EDTA, 10% glycerol, 0.5% NP40, 0.25% Triton-X100], rotated for 10min at 4°C, spun at 1600g for 5min, resuspended in ice-cold 1ml buffer LB2 [10mM Tris pH 8.0, 200mM NaCl, 1mM EDTA, 0.5mM EGTA], rotated for 10min at 4°C, spun at 1600g for 5min and resuspended in 140 μ l shearing buffer [1% SDS, 10mM EDTA, 50mM Tris pH 8.0]. Nuclear Lysates were sonicated with Bioruptor (Diagenode) at High setting, for 26 cycles (30sec ON / 30sec OFF) to obtain DNA fragments with an average size of 300bp. Lysates were spun in a microcentrifuge at 8°C at maximum setting for 10mins to remove debris. 130 μ l of supernatant (equivalent of approximately 5×10^6 cells) was diluted in 1300 μ l 11x dilution buffer [50mM Tris pH 8.0, 167mM NaCl, 1.1% Triton X-100, 0.11% Na-Deoxycholate]. Lysates were frozen at -80°C at this point or processed immediately. 1430 μ l diluted nuclear lysate (equivalent of approximately 5×10^6 cells) was pre-cleared by incubating with 2-4 μ g of isogenic normal IgG and 25 μ l Protein G Dynabeads (Invitrogen, 1004D) on a rotator at 4°C for 2 hrs. Supernatants were then incubated with the appropriate ChIP antibody (see Table 6 for dilutions) on a rotator overnight at 4°C, followed by incubation with 30 μ l Protein G Dynabeads for 1h at 4°C. To remove unbound chromatin and unspecific interactions, beads were washed two times with Wash Buffer 1 [50mM Tris pH 8.0, 0.1% SDS, 0.1% Na-Deoxycholate, 1% Triton X-100, 150mM NaCl, 1mM EDTA, 0.5mM EGTA], one time with Wash Buffer 2 [50mM Tris pH 8.0, 0.1% SDS, 0.1% Na-Deoxycholate, 1% Triton X-100, 500mM NaCl, 1mM EDTA, 0.5mM EGTA], one time with Wash Buffer 3 [50mM Tris pH 8.0, 250mM LiCl, 0.5% Na-Deoxycholate, 0.5% NP-40, 1mM EDTA, 0.5mM EGTA] and two times with Wash Buffer 4 [50mM Tris pH 8.0, 10mM EDTA, 5mM EGTA]. Each wash was performed for 5mins on a rotator using ice-cold buffers and protease/phosphatase inhibitors (Roche). Chromatin-antibody complexes were eluted by incubating in 125 μ l of Elution Buffer [1% SDS, 0.1M NaHCO₃] on a shaker block at 37°C for 15mins, repeating twice. Elutions were pooled and DNA was purified using Qiagen Minelute PCR purification kit.

The following antibodies and cell numbers were used for each ChIP replicate: H3K4me3 ChIP, 3×10^6 cells, 3 μ g rabbit H3k4me3 antibody (Diagenode pAb-MEHAHS-024, A1-010); H3K27Ac ChIP, 3×10^6 cells, 2 μ g rabbit H3K27Ac antibody (Active Motif, 39159); RBPJ ChIP, 5×10^6 , 4.5 μ L polyclonal rabbit RBPJ antibody (Cell Signaling Technology, 5442S), Etv5-3xFlag ChIP; 5×10^6 cells, 3 μ g anti-Flag M2 antibody (Sigma-Aldrich). For H3K27Ac and RbpJ ChIP, 2 μ g normal rabbit IgG (Abcam, ab171870) was used for pre-clearing step and a different batch of rabbit IgG (Cell Signaling Technology, 2729S) was used for negative control samples. Etv5-Flag ChIP was performed on lysates obtained from two independently derived clonal ES cell lines (Etv5-Flag-KI-20 and Etv5-Flag-KI-32) that carry a 3xFlag epitope knocked in to the C-terminal end of the endogenous *Etv5* coding sequence just before the stop codon. Parental RGd2 ES cells served as negative control. ChIP-Seq libraries were generated using NEB Next ChIP-Seq Library Prep Master Mix Set for Illumina (NEB).

ChIP-seq analysis

Sequencing reads were mapped to the mm10 mouse reference genome using Bowtie2 (Langmead and Salzberg, 2012), converted to a density plot and displayed as UCSC genome browser custom tracks. ETV5 peaks were called over RGd2 parental line controls using MACS2 software (Zhang et al., 2008). Mapped reads were converted to density plots and displayed as UCSC genome browser custom tracks. Only the peaks called in both biological replicates ($p < 10^{-4}$) were selected for further analyses. Peaks overlapping a promoter (1000 \pm RefSeqGene TSS coordinates) by at least 1bp, or a gene body was assigned to only that gene. The rest of the peaks (intergenic peaks) were assigned to the nearest genes within 50Kb. To identify potential direct targets of Etv5, the peaks were intersected with genes that show a fold change of ≥ 1.5 in the UP direction and ≤ 0.66 in the DOWN direction in Etv5-KO ESCs over parental RGD2 ESCs.

H3K27ac, H3K4me1, p300 and input data from Buecker et al, 2014 with accession number GSE56138 were downloaded from the NCBI Gene Expression Omnibus. The reads were aligned to the mm10 reference genome using bowtie (-y -m 1 --best --strata --nomaqround) and converted to bigwig using deeptools 6 (Ramirez et al., 2016) bamCoverage (--extendReads 200 --binSize 1 --normalizeTo1x 2150570000). The mean signal at the ETV5 peaks was extracted using Deeptools computeMatrix using the ETV5 peak centers as reference points.

Transcriptome sequencing (RNA-seq)

RNA-seq was performed in triplicates per condition, including three independently derived clonal lines per genetic knock-out, and two RGd2 lines as wild type controls. Exception is RGd2-N16h samples for which only 2 replicates were sequenced, as one was lost during library preparation. RGd2-2 is a clonal line derived from the parental RGd2 line (RGd2-1). Total RNA was extracted with ReliaPrep RNA Miniprep System (Promega) and was processed with Ribo-Zero capture probes (Illumina). Libraries were produced using NEXTflex Rapid Directional qRNA-Seq Kit (Bioo Scientific). Libraries were sequenced in the Illumina platform in paired-end mode.

RNA-seq analysis

Illumina sequencing adapters were removed using Trim Galore! and reads shorter than 20 nt were discarded. The reads were aligned to the mouse reference genome (GRCm38/mm10) with ERCC spike-ins appended to it. The alignment was done using TopHat2 and Gencode (release M14) gene models were used as a guide. Read counts per gene were calculated using featureCounts requiring strand-specific, primary and unique matches. Normalization and statistical analysis of the resulting counts table was done using the R Bioconductor

package DESeq2 using normalization factors based on the spike-in counts. Gene counts were converted to fragments per kilobase per million mapped reads (FPKM) and then log₂-transformed for visualization in heatmaps and MA-plots. A significance threshold of $\text{padj} < 0.05$ and $\text{FPKM} \geq 1$ was used to define differentially expressed genes.

KEGG pathway analysis:

Enriched KEGG pathways were identified using the 'goseq' package from R Bioconductor. Only expressed genes with mean FPKM ≥ 1 were considered. Differentially expressed genes with $\text{padj} < 0.05$ and log₂ fold change > 0.5 or < -0.5 were compared with all expressed genes. False discovery rate was calculated to correct for multiple testing using the 'p.adjust' function in R.

Cluster analysis and PCA:

Regularized log-transformed counts were calculated using DESeq2 and used for sample clustering and PCA. Hierarchical clustering was done using the 'pheatmap' package in R with sample distances calculated by the 'dist' function. PCA was done by the 'prcomp' function without scaling.

Data and Software Availability

ChIP-seq and RNA-seq data from this study have been submitted to GEO database with the accession number GSE122338.

Chimaera production

Pregnant females were killed on day 2.5 post coitum (E2.5) by cervical dislocation. Oviduct and uterus were dissected, and flushed with M2 media (Sigma, M7167) using Leica M165C microscope system for better visualization. Embryos were collected in M2 media prior to microinjection. E2.5 embryos were transferred into M2 media, covered with a layer of mineral oil (Sigma, M8410). Embryos were visualized using an Olympus microscope system and an Olympus 40x LWD Plan APO 0.6 NA air objective. ESC were loaded into a microinjection pipette and injected into the perivitelline space of 8-cell embryos using Hamilton Thorne XYClone microinjection system (Hamilton Thorne). Eight cells were transferred into each embryo. Injected embryos were cultured for 2 days in BlastTM media (Origio, 8306001A) at 37 °C, 20% O₂ and 7% CO₂. For post-implantation analyses, embryos were transferred one day after injections into oviducts of pseudopregnant females. Contribution was characterised at E4.5, E6.5 and E7.5.

Immunofluorescence staining of embryos

For immunofluorescence analysis of cultured pre-implantation stage embryos, zona pellucidae were removed using tyrode acid solution (pH 2.5). Embryos were fixed with 4% w/v paraformaldehyde (PFA) (Sigma, P6148) in PBS for 15 minutes at room temperature (RT). Samples were washed three times with PBS, supplemented with 3 mg/ml poly(vinylpyrrolidone) (PVP) (Sigma, P0930) (PBS/PVP). For permeabilization, embryos were incubated for 30 minutes in 0.25% Triton-X-100 (Sigma-Aldrich, T8787) diluted in PBS/PVP. Embryos were incubated for 15 minutes in 2% donkey serum, 0.25% bovine serum albumin (BSA) (Sigma, 1076192), 0.01% Tween 20 (Sigma Aldrich, P2287) in PBS, followed by overnight incubation in primary antibodies diluted in blocking buffer (Table S6). On the next day embryos were washed three times for 15 minutes in blocking buffer before incubation for 1h in secondary antibodies diluted in blocking buffer. Afterwards, embryos were washed three times for 15 minutes in blocking buffer with or without DAPI.

Dissected post-implantation stage embryos were fixed for 1 hour in 4% PFA. Embryos were washed three times 15 minutes in PBS/PVP. For permeabilization, embryos were incubated for 1 hour in PBS containing 5% DMSO (Santa Cruz, sc-358801), 0.5% Triton-X-100, 0.1% BSA and 0.01% Tween20 at 4 °C. Embryos were blocked overnight at 4 °C in humidified

environment in permeabilization buffer, containing 2% donkey serum. On the next day embryos were incubated overnight at 4°C in primary antibodies (Table S7) in blocking buffer. Embryos were washed 3 times for 2 hours in blocking buffer, before incubation overnight in secondary antibodies diluted in blocking buffer. Lastly, embryos were washed three times for 2 hours in blocking buffer with or without DAPI.

For embryo mounting, samples were taken through a series of 25%, 50%, 75% and 100% vectashield (Vector Laboratories, H-1000) diluted in PBS. Embryos were mounted in a drop of vectashield, surrounded by drops of Vaseline as a spacer for the coverslip, to immobilise embryos. Coverslips were sealed using nail varnish. Finally, slides were stored at -20°C prior to imaging.

Embryos were imaged using a Leica TCS SP5 confocal microscope. Image acquisition was performed with a 20x 0.7NA air objective. For illumination, a 405, 488, 561 and 647 nm lasers and Leica application suite was used. Images were processed with Fiji software.

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KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
pERK p44/42 MAPK (T202/T204) XP®	Cell Signaling Technology	Cat#4370S
Etv5	Abcam	Cat#ab102010, RRID:AB_10711030
Flag M2	Sigma-Aldrich	Cat#F1804, RRID:AB_262044
Rbpj	Cell Signaling Technology	Cat#5442S, RRID:AB_10695407
Normal rabbit IgG	Cell Signaling Technology	Cat#2729S
Normal rabbit IgG	Abcam	Cat#ab171870
Normal mouse IgG	Santa Cruz	Cat#sc-2025
GAPDH	Sigma-Aldrich	Cat#G8795, RRID:AB_1078991
H3K27Ac	Active Motif	Cat#39135, RRID:AB_2614979
ERK	Cell Signaling Technology	Cat#9107
Pou3f1 (Oct6) C-20	Santa Cruz	Cat#sc-11661
Lef1	Abcam	Cat#ab137872
GATA4	Santa Cruz	Cat#sc1237, RRID: AB_2108747
Sox2	eBioscience	Cat#14-9811-80, RRID: AB_11219070
Tuj1	R&D Systems	Cat#MAB1195
Sox1	Cell Signaling Technology	Cat#4194, RRID:AB_1904140
Klf4	R&D Systems	Cat#AF3158, RRID:AB_2130245
Nanog	eBioscience	Cat#14-5761-80, RRID:AB_763613
Tfcp2l1	R&D Systems	Cat#AF572, RRID:AB_2202564
H3K4me3	Diagenode	Cat#pAb-MEHAHS-024
GFP	ThermoFisher Scientific	Cat#A-11122, RRID:AB_221569
T (Brachyury)	R&D Systems	Cat#AF2085, RRID:AB_2200235
Oct4	Santa Cruz	Cat#sc-5279, RRID:AB_628051
b-tubulin	Abcam	Cat#ab6046, RRID:AB_2210370
Bacterial and Virus Strains		
N/A		
Biological Samples		
N/A		
Chemicals, Peptides, and Recombinant Proteins		
MEK inhibitor PD0325901	ABCR	Cat#AB 253775
GSK3 inhibitor CHIR99021	ABCR	Cat#AB 253776
Laminin	Millipore	Cat#CC095-5MG
Fibronectin	Millipore	Cat#FC010
ROCK inhibitor Y-27632	Merck Chemicals	Cat#688000-100MG
Tankyrase inhibitor XAV939	Cell Guidance Systems	Cat#SMS38-200
Activin A	Made in house	N/A
Fgf2	Made in house	N/A
LIF	Made in house	N/A
N2B27	Made in house	N/A

Accutase	Millipore	Cat#SCR005
Gelatin	Sigma-Aldrich	Cat#G1890
Γ-secretase inhibitor	Calbiochem	Cat#CAS 209984-56-5
Γ-secretase inhibitor DAPT	Merck Chemicals	Cat#565770
FuGENE HD transfection reagent	Promega	E2311
Critical Commercial Assays		
Alkaline Phosphatase Kit	Sigma-Aldrich	Cat#86R-1KT
NEPER Nuclear Cytoplasm Extraction Reagents	ThermoFisher Scientific	Cat#78833
Dynabeads Protein G	Thermofisher Scientific	Cat#10004D
Deposited Data		
Etv5 ChIP-seq (2i, N16h)	This study	GSE122338
H3K4me3 ChIP-seq (2i, N16h)	This study	GSE122338
RNA-seq of Etv5, Rbpj and Tcf3 single, double and triple mutant ESCs	This study	GSE122338
Experimental Models: Cell Lines		
Rex1-GFPd2 (RGd2) ESC	Kalkan et al, 2017	N/A
Rex1-GFPd2 c1 (RGd2-2) ESC (clonal line)	This study	N/A
Etv5 knock-out ESC (3 clonal lines)	This study	N/A
Rbpj knock-out ESC (3 clonal lines)	This study	N/A
Tcf3-knock-out ESC (2 clonal lines)	This study	N/A
Etv5/Tcf3 double knock-out ESC (2 clonal lines)	This study	N/A
Rbpj/Tcf3 double knock-out ESC (2 clonal lines)	This study	N/A
Etv5/Tcf3/Rbpj triple knock out ESC (3 clonal lines)	This study	N/A
Etv4 knock-out ESC (3 clonal lines)	This study	N/A
Etv4/Etv5 knock-out lines (1 clonal line)	This study	N/A
Hes1 knock-out ESC (2 clonal lines)	This study	N/A
Id3 knock-out ESC (2 clonal lines)	This study	N/A
Lef1 knock-out ESC (2 clonal lines)	This study	N/A
Pou3f1 knock-out ESC (2 clonal lines)	This study	N/A
Pou3f1/Lef1 double knock-out ESC (2 clonal lines)	This study	N/A
RGd2/mKusabira Orange (mKO) ESC	This study	N/A
RGd2/ H2B-tdTomato ESC	This study	N/A
ETR-tKO/mKusabira Orange(mKO) ESC	This study	N/A
ETR-tKO/H2B-tdTomato ESC	This study	N/A
Etv5-3xFlag knock-in ESCs (2 clonal lines)	This study	N/A
Etv5-KO/Etv5-3xFLAG rescue ESC	This study	N/A
Etv5-KO/ΔEtv5-3xFLAG rescue ESC	This study	N/A
Etv5-KO/mKusabira Orange (mKO)	This study	N/A
Rbpj-KO/Rbpj rescue ESC	This study	N/A

ETR-tKO/TetG/TRE3G-iEpT ESC	This study	N/A
ETR-tKO/TetG ESC	This study	N/A
Etv4/Etv5 double knock-out ESC (2 lines; PE15-3, PE15-4)	Lu et al, 2009	N/A
Etv4/Etv5 dKO (PE3/PE4)/ mKusabira Orange ESC	This study	N/A
Etv4/Etv5 dKO (PE3/PE4)/ Etv5-3xFlag rescue ESC	This study	N/A
Etv4/Etv5 dKO (PE3/PE4)/ ΔEtv5-3xFLAG rescue ESC	This study	N/A
Wild type ESC	Lu et al, 2009	N/A
Experimental Models: Organisms/Strains		
Mus musculus females aged 6-10 weeks: CD1 strain was used to provide embryos and CBA/BL6 F1 animals were employed as transfer recipients for embryo chimaeras.	N/A	N/A
Oligonucleotides		
Nanog siRNA, Mm_Nanog_3 FlexiTube siRNA	Qiagen	Cat#SI01323357
Nanog siRNA, Mm_LOC100038891_2 FlexiTube siRNA	Qiagen	Cat#SI04460869
Tbx3 pre-designed siRNA Assay ID: 223884	Thermofisher Scientific	Cat#AM16708
Tbx3 pre-designed siRNA Assay ID 223885	Thermofisher Scientific	Cat#AM16708
gRNAs Sequences	See Table S7 for sequences	
Taqman and UPL gene expression assays	See Table S7 for oligo sequences and catalog numbers	
ChIP-qPCR Primer Sequences	See Table S7 for sequences	
Recombinant DNA		
TRE3G-Etv5-p2A-Tcf3-pGK-Hygro	This study	N/A
CAG-Etv5-3xFlag-pGK-Hygro	This study	N/A
CAG-ΔN-Etv5- 3xFlag-pGK-Hygro	This study	N/A
CAG-mKusabira Orange-pGK-Hygro	This study	N/A
CAG-Rbpj-pGK-Hygro	This study	N/A
CAG-H2B-tdTomato-IRES-Puromycin	This study	N/A
px459_SpCas9-2A-Puro	Addgene	#62988
Software and Algorithms		
Bowtie2	Langmead and Salzberg, 2012	
MACS2	Zhang et al., 2008	
DeepTools	Ramírez et al., 2016	doi: 10.1093/nar/gkw257

Trim Galore!	Felix Krueger, 2015	https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/
Bowtie2	Langmead and Salzberg, 2012	http://bowtie-bio.sourceforge.net/bowtie2/index.shtml
Samtools	Li et al., 2009	http://samtools.sourceforge.net/
FeatureCounts	Liao et al., 2014	doi: 10.1093/bioinformatics/btt656
R	R Core Team, 2017	https://www.R-project.org/
DESeq2	Love et al., 2014	https://www.bioconductor.org/packages/release/bioc/html/DESeq2.html
Goseq	Young et al., 2010	https://www.bioconductor.org/packages/release/bioc/html/goseq.html
Pheatmap	Raivo Kolde, 2015	https://CRAN.R-project.org/package=pheatmap
Other		
N/A		

Figure 1

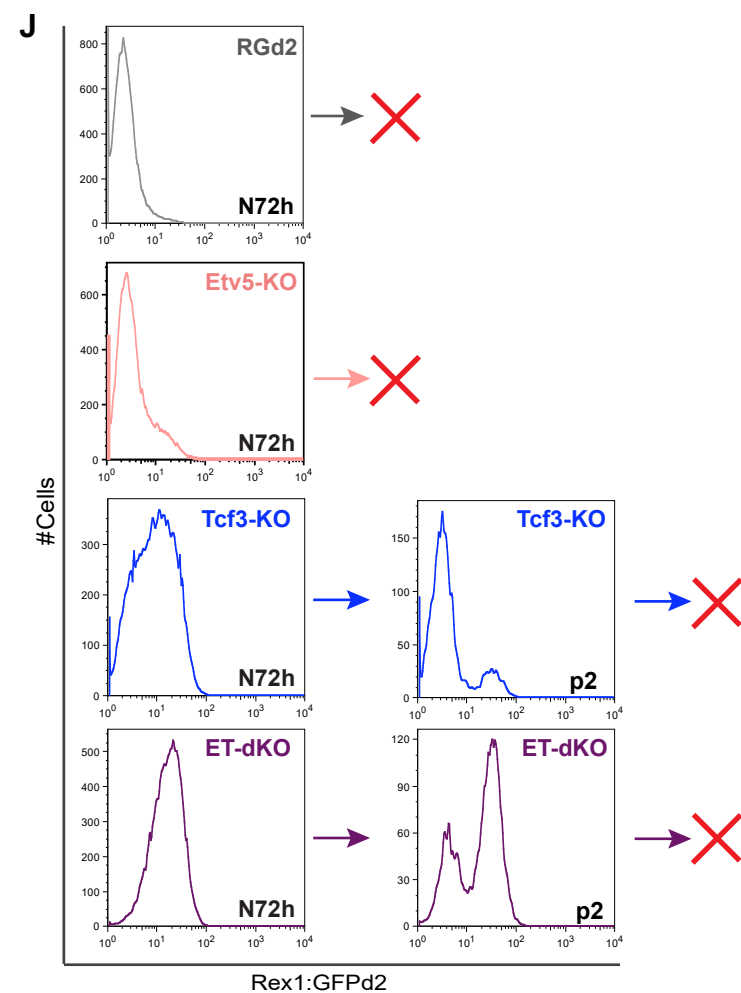
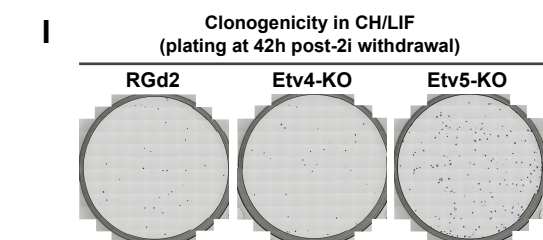
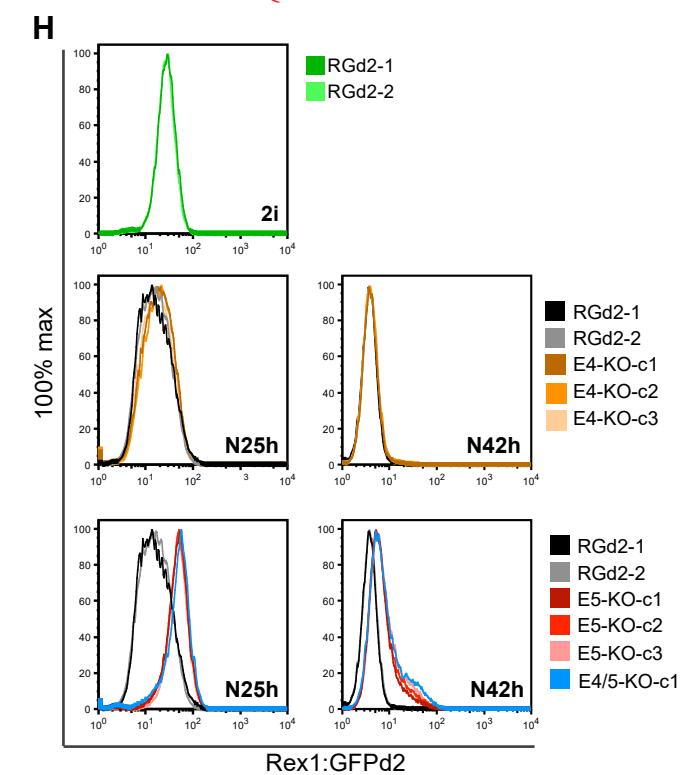
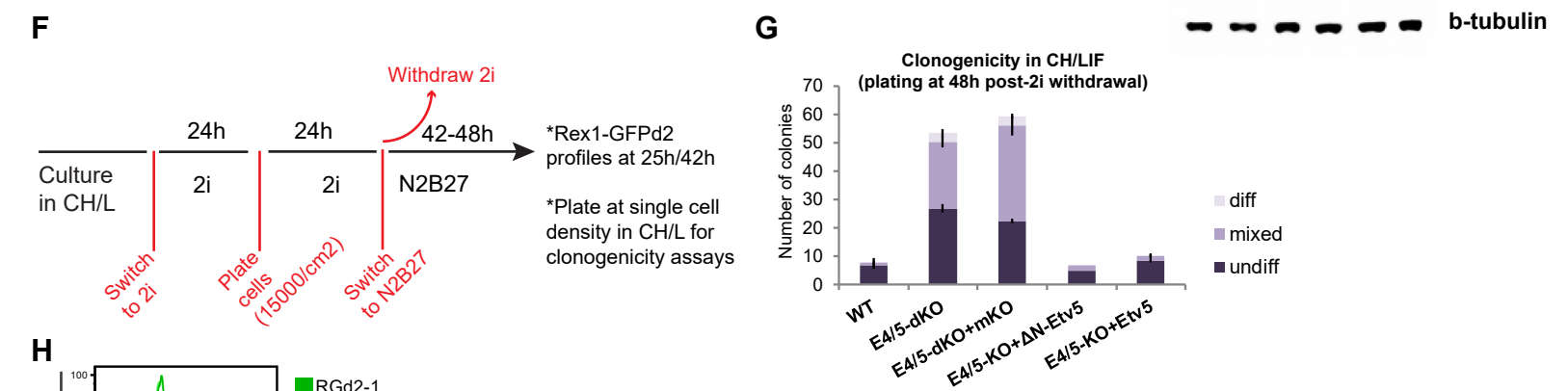
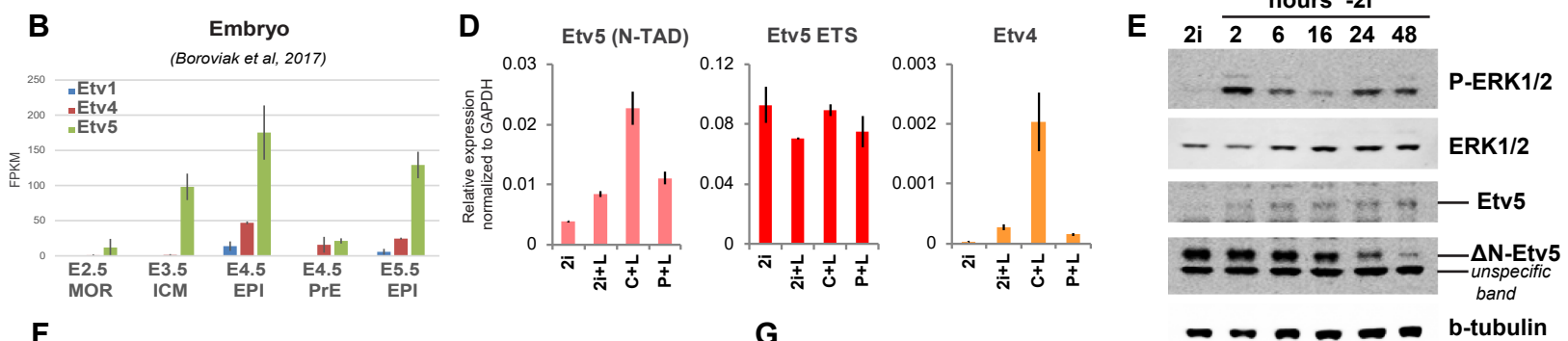
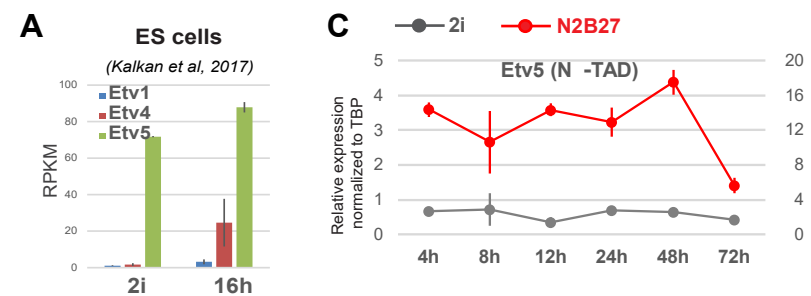


Figure 2

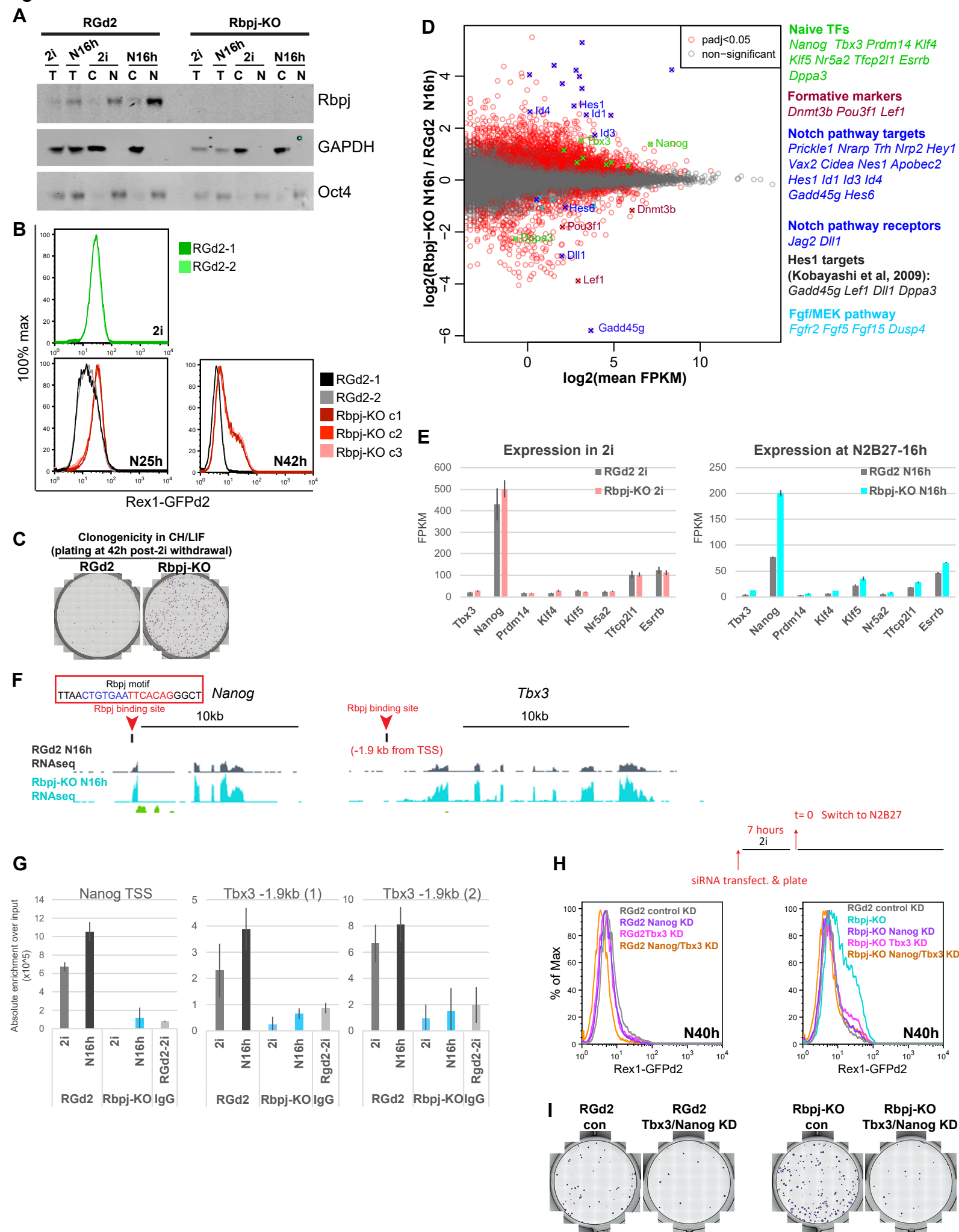


Figure 3

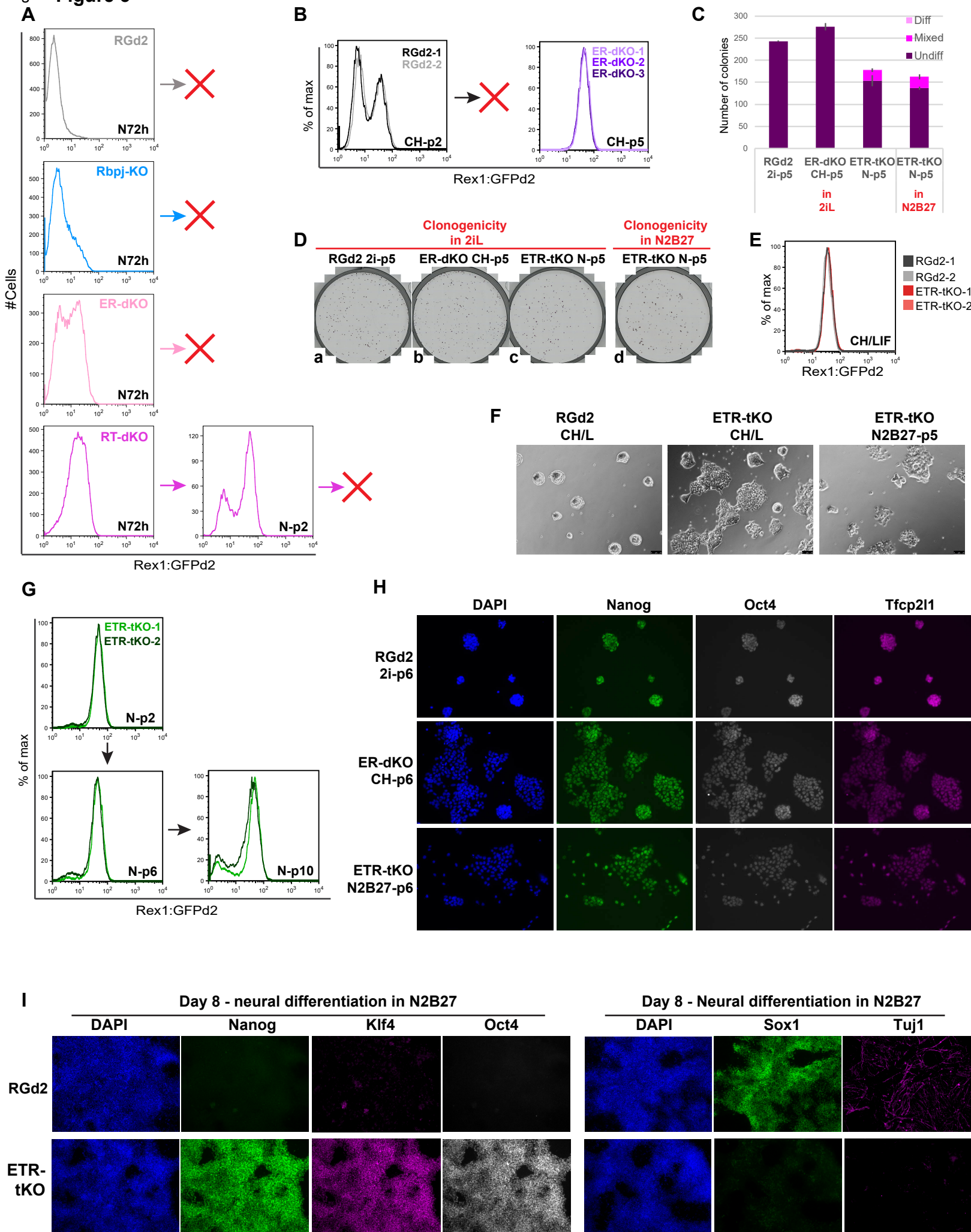


Figure 4

Fig 4

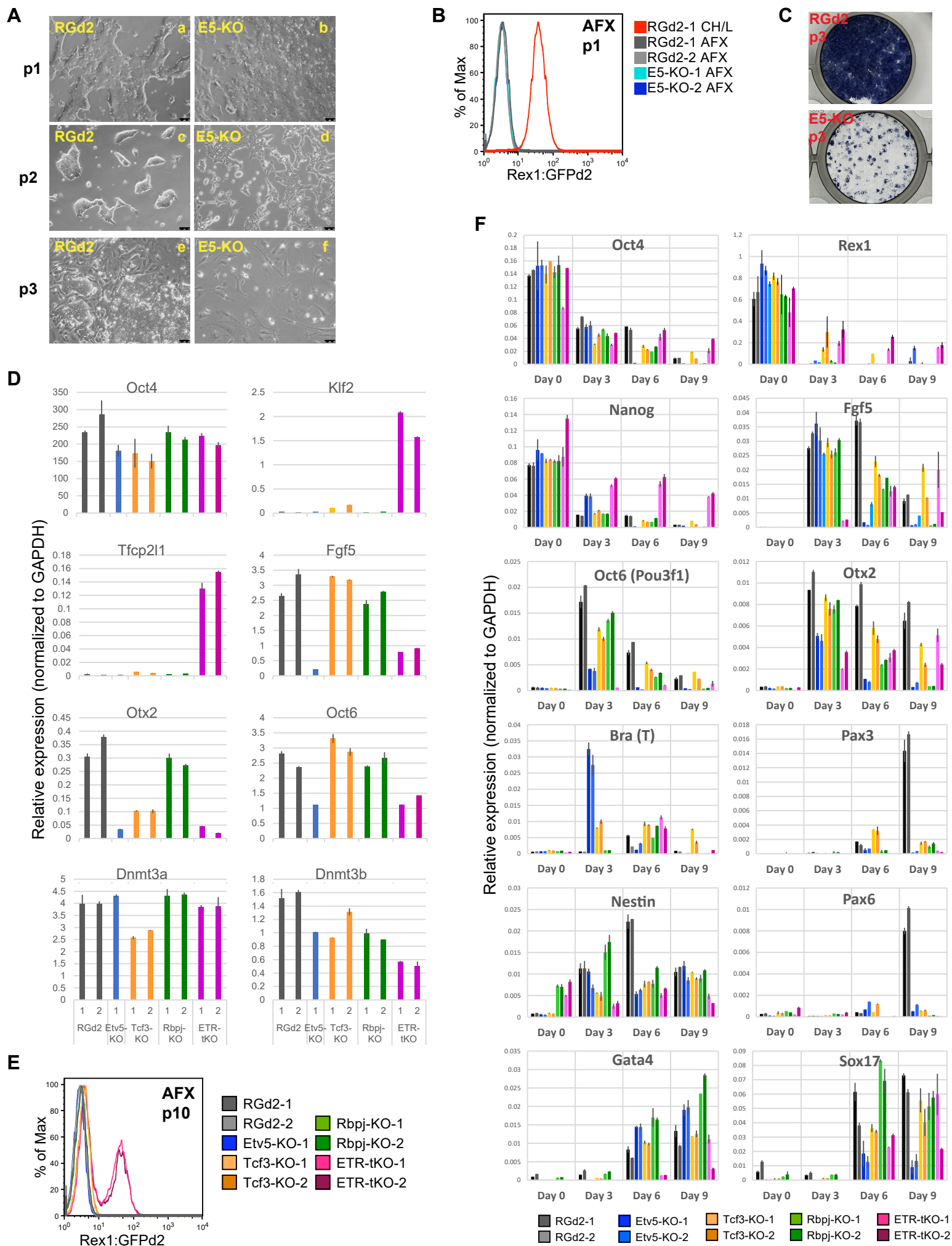


Fig 5

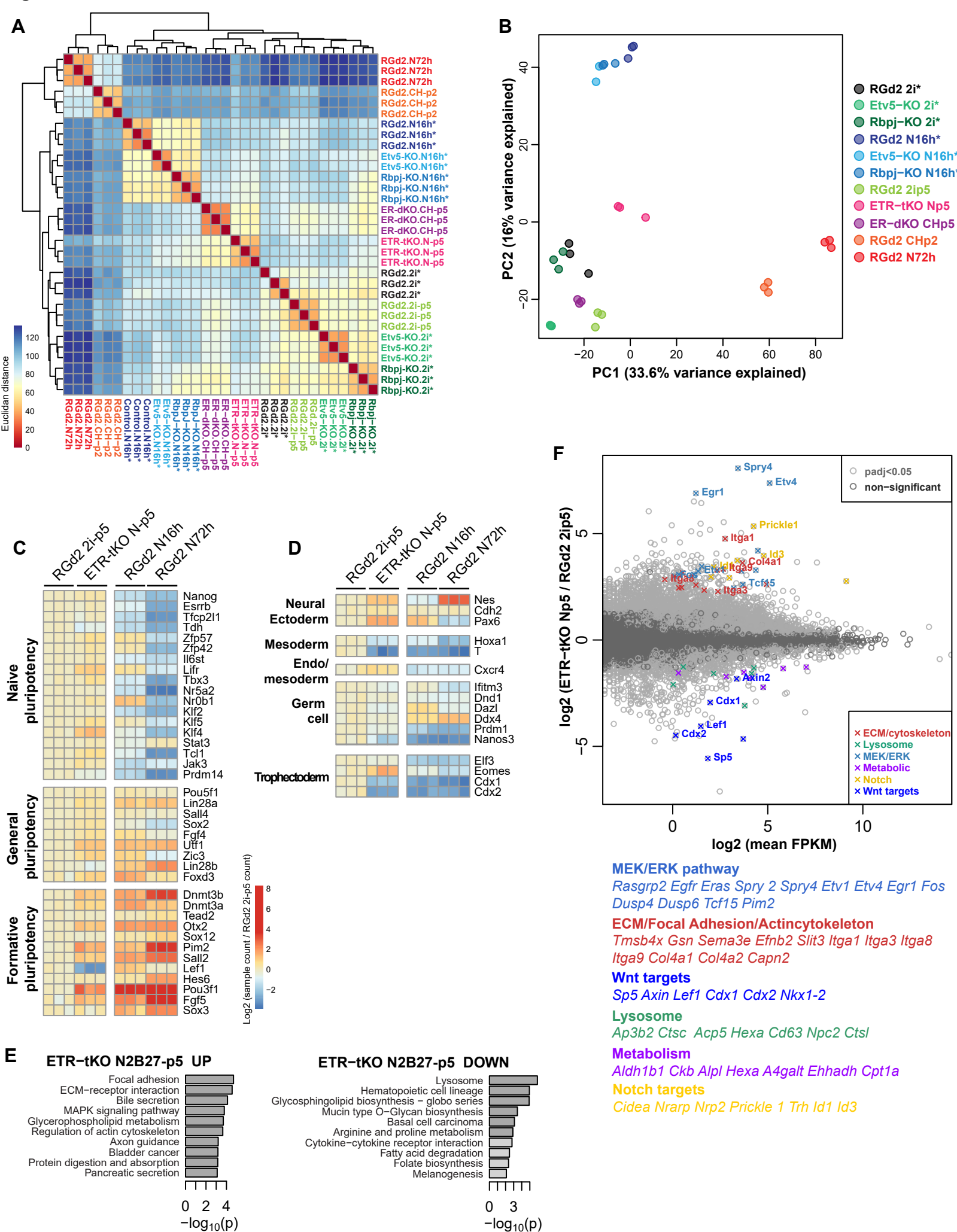


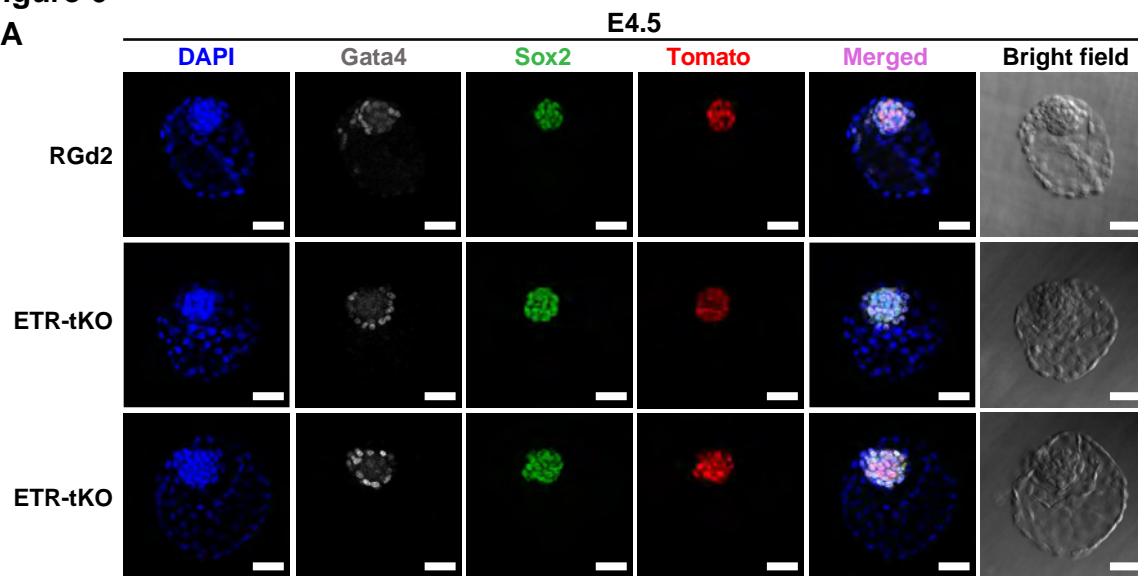
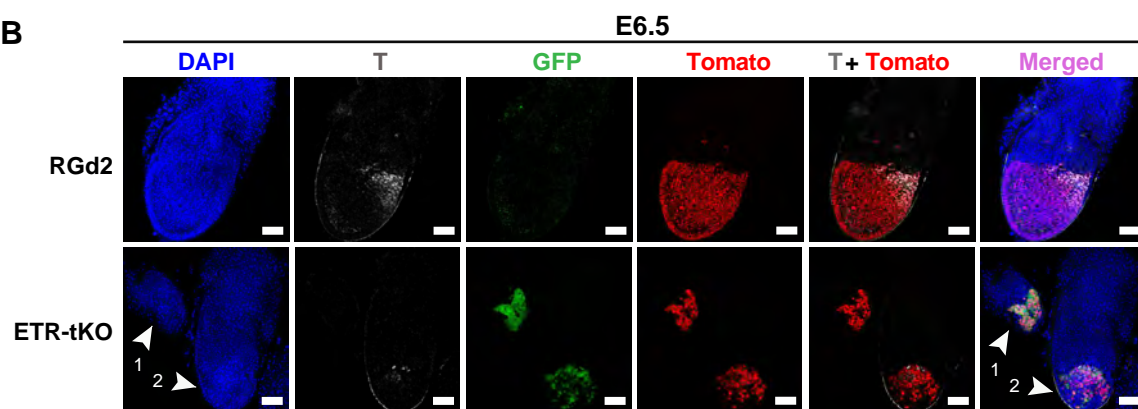
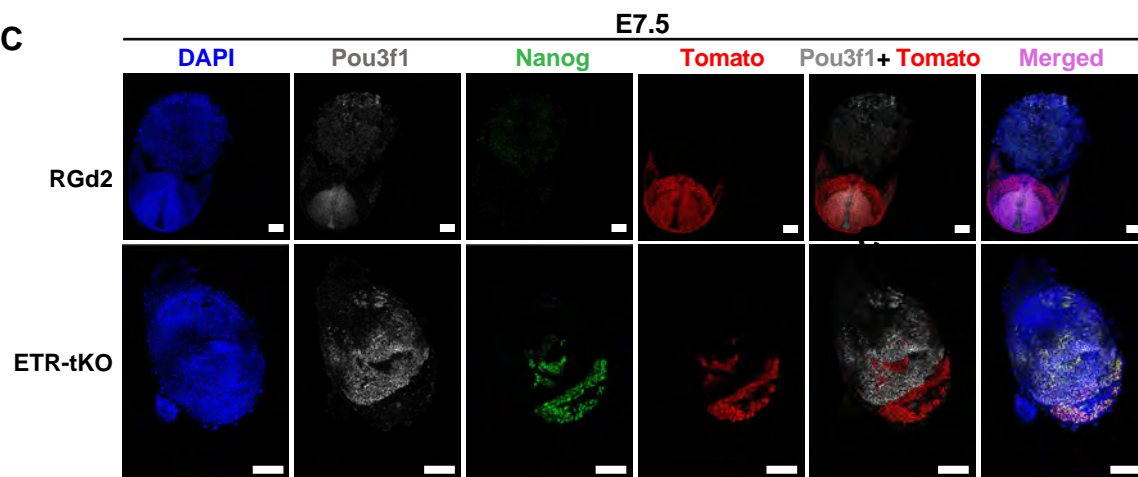
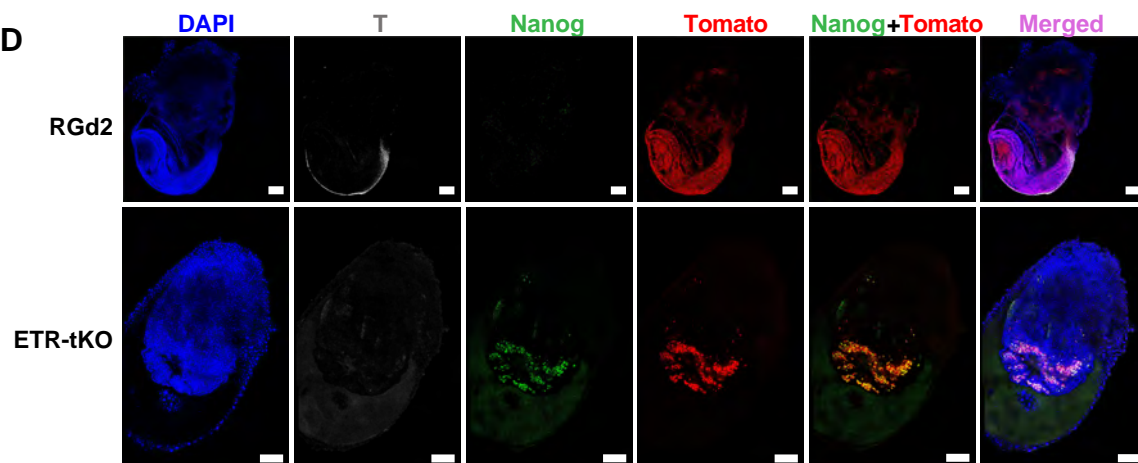
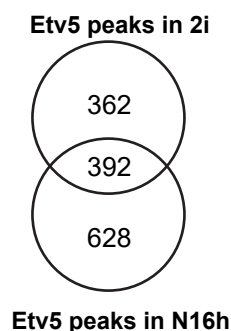
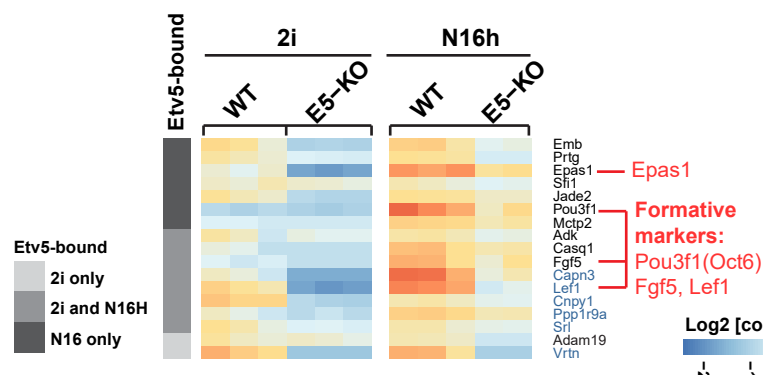
Figure 6**A****B****C****D**

Figure 7

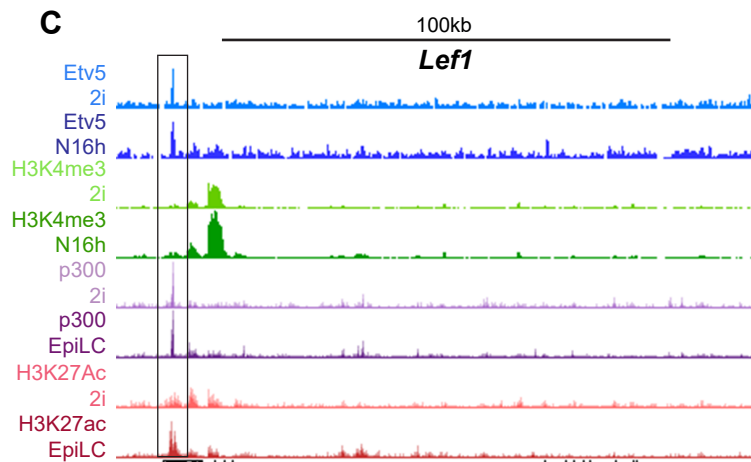
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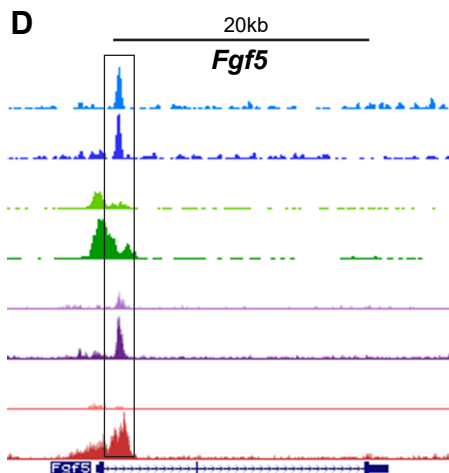
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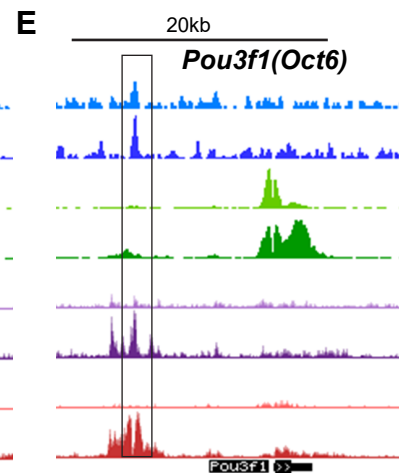
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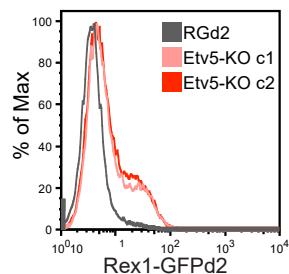
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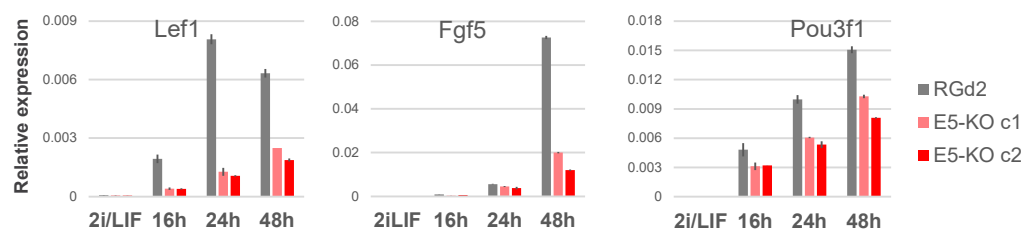
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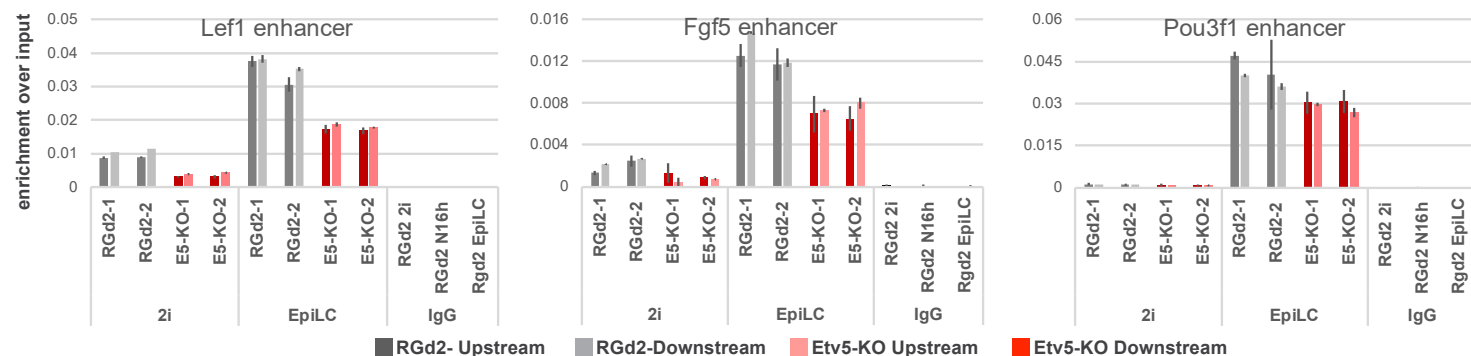
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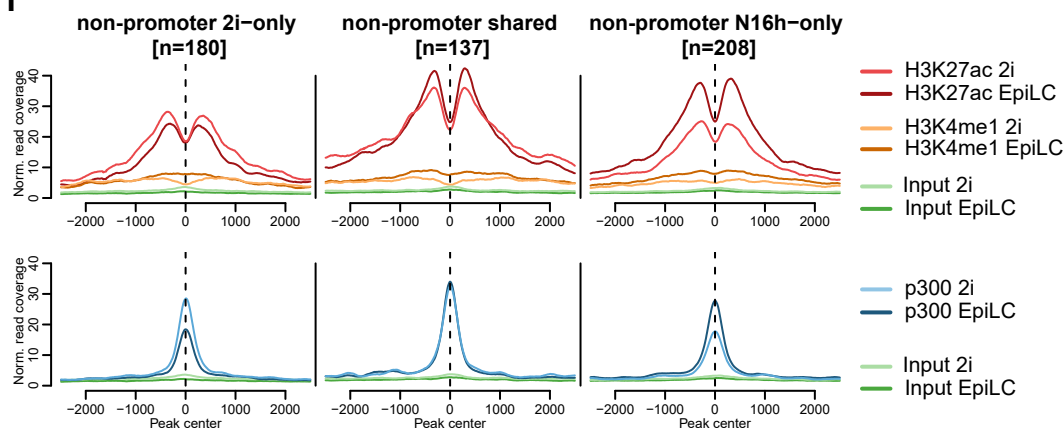
G



H



I



Supplemental Figure Legends

Figure S1. ETV4/5 expression and function (Related to Figure 1)

(A) RNA-seq (data from Kalkan et al, 2017) and H3K4me3 ChIP-seq (this study) genome tracks for *Etv5*.

(B) Western blot with polyclonal ETV5 antibody for detection of endogenous ETV5 in RGd2 ESC. 2i cultures (0h) were switched to fresh 2i (2i), N2B27 (N) or single inhibitors (CH) or (PD) for 16 hours. Cells were transfected with negative control siRNA or siRNA against *Etv5*.

(C, D) Relative growth rates of: wildtype and *Etv4/5*-dKO ESC from Lu et al, 2009; RGd2 and *Etv5*-KO ESC lines. Cell numbers are normalized to WT for panel C, and to parental RGd2-1 ESC for panel D. Error bars indicate standard deviation (SD) from 2 wells cultured in parallel.

(E) Restoration of Rex1-GFPd2 downregulation kinetics in two *Etv5*-KO clonal ESC lines carrying stable transgenes encoding ΔN and canonical isoforms of ETV5.

(F) RT-qPCR for ETV4 in 2i and at indicated hours after 2i withdrawal. Error bars indicate SD from two wells cultured in parallel.

Figure S2. RBPJ expression and function (Related to Figures 1 and 2)

(A, B) TCF3 transcript levels in RGd2 and *Etv5*-KO ESCs in 2i and at hours (h) post-2i withdrawal.

(C-F) RBPJ transcript levels in: the early mouse embryo; RGd2 and *Etv5*-KO ESCs in 2i and hours (h) after 2i withdrawal; RGd2 and mutant ESCs cultured in CH/L and 28h after CH/LIF withdrawal. Error bars indicate SD from 2 or 3 biological replicates for RNA-seq, or from 2 wells cultured in parallel for RT-qPCR.

(G) Phase contrast images of parental, *Rbpj* KO and transgene rescued ESC; MT=empty vector, *Rbpj* tg=transgene encoding RBPJ. Scale bar= 0.75 μ m

(H) Growth rates of two different RGd2 and *Rbpj*-KO ESC lines. All cell numbers were normalized to RGd2-1 ESC line.

(I) Restoration of Rex1-GFPd2 downregulation in *Rbpj*-KO ESC line stably expressing *Rbpj* transgene.

(J, K) Transcript levels of Notch target genes, and Notch pathway components.

(L) GFP profiles of RGd2 ESCs treated with Notch inhibitors (iNotch) or DMSO: (1) before 2i withdrawal; (2) after 2i withdrawal; (3) before and after 2i withdrawal.

Figure S3. RBPJ expression and function (Related to Figure 2)

(A, B) KEGG pathway enrichment for differentially expressed genes in *Rbpj* mutants vs. parental RGd2 ESC in 2i, and in N16h.

(C) Relative expression of genes associated with ECM and cell adhesion identified by KEGG pathway enrichment analysis.

(D) MA-plot showing mean expression against fold change per gene in *Rbpj*-KO ESC in 2i. Gene symbols are shown for indicated genes.

(E) Heatmaps showing relative expression of TOP 50 differentially expressed genes with mean FPKM \geq 1 in *Rbpj*-KO N16h for UP genes, and FPKM \geq 1 in RGd2 N16h for DOWN genes. Genes were ordered according to fold change of mean RNA-seq count in *Rbpj*-KO N16h over RGd2 N16h.

Figure S4. RBPJ expression and function (Related to Figures 2 and 3)

- (A)** Expression of HES1 and ID factors in *Rbpj* mutants. Error bars show SD for 3 independent clonal lines for *Rbpj*-KO, and 2 different lines for RGd2 (one parental, one clonal) cultured in parallel.
- (B, C)** Rex1-GFPd2 profiles of Id3 knock-out, and *Hes1* knock-out ESC. Data are shown for independent clonal lines cultured in parallel.
- (D)** ChIP-qPCR for RBPJ-bound loci identified by Lake et al, 2014. *Hes1* TSS and gene desert sites were used as positive and negative control regions, respectively. *Rbpj*-KO ESCs were used as negative control. Values were normalized to input DNA. Asterisks mark enrichment in *Nanog* and *Tbx3* promoter proximal sites. Error bars indicate SD from 2 qPCR replicates.
- (E)** GFP profiles at 29h after 2i withdrawal following a 7-hour period of siRNA transfection.
- (F)** Venn diagram showing the overlap of upregulated genes in *Rbpj*-KO ESCs at 16h post-2i withdrawal from RNA-seq (this study) and RBPJ-associated genes from published ChIP-seq dataset. Only genes that show a fold change of ≥ 1.5 increase in *Rbpj*-KO over RGd2 are considered. Pluripotency regulators are shown in boxes and naïve pluripotency transcription factors are in bold.
- (G)** Venn diagram showing intersection of potential targets repressed by RBPJ and TCF3.
- (H)** GFP profiles of parental RGd2 ESC, *Etv5/Rbpj* single and double mutants in CHIR99021 (CH) only. Crossed box indicates failure of expansion after passage 2 (p2).
- (I)** Growth rates of RGd2 ES cells in 2i, *Etv5/Rbpj* single and double mutants in CH. Error bars indicate SD from two independently-derived lines cultured in parallel.
- (J)** Colony formation by RGd2 and ER-dKO ES cells in CH or CH/LIF after culture for 2 passages (p2) in CH only. Zoomed-in images show larger colonies from ER-KO ESCs.

Figure S5. Dual and triple knock out phenotypes (Related to Figure 3)

- (A)** Bright field images of mutant lines taken using a 20x objective.
- (B, C)** Relative growth rates of indicated ESC over 5 passages in CH/L, and of RGd2 in 2i or ETR-tKO in N2B27. Independently derived ESC lines are numbered (1-3).
- (D)** Flow cytometry profiles (**a, c, e**) of mixed cultures comprised of unlabelled RGd2 ESCs and mKO-labelled RGd2 or mKO-labelled ETR-KO ESCs, and corresponding GFP profiles (**b, d, f**) of unlabelled RGd2 ESCs gated out from mixed cultures with mKO RGd2 (gray) or mKO ETR-KO ESC (light red).
- (E)** GFP profiles for ETR-KO cells with Doxycycline inducible *Etv5*-p2A-Tcf3 (iEp2aT) transgene expression.
- (F)** RT-qPCR at 52h post-2i withdrawal and Doxycycline (dox) addition (100ng/ml). Error bar show SD from 2 qPCR replicates.
- (G)** IF images for NANOG at 3 days post-2i withdrawal and Doxycycline (dox) addition (100ng/ml) taken using a 10x objective.

Figure S6. ETV5 association with transcriptionally dynamic genes (Related to Fig 7)

- (A)** Distribution of unique ETV5 ChIP-seq peaks in 2i and N16h samples. See STAR Methods for peak selection and classification.
- (B)** Heatmap for relative expression of genes associated with ETV5 that are up- and downregulated in 2i.
- (C)** Heatmap for ETV5 associated genes upregulated at 16h post-2i withdrawal (N16h). Only genes that show a fold change of ≥ 1.5 in the UP direction and ≤ 0.66 in the DOWN direction

in *Etv5*-KO over RGd2 are considered. Expression shown as the log2 difference to the mean across all samples.

(D) Venn diagrams showing the intersection of TCF3 and RBPJ targets with ETV5-bound, upregulated genes in *Etv5*-KO ESCs. Pluripotency regulators are shown in boxes and naïve pluripotency factors are in bold case.

Figure S7. ETV5 association with transcriptionally dynamic genes (Related to Fig7)

(A) RT-qPCR for *Otx2* in parental RGd2 and *Etv5*-KO ESCs upon 2i withdrawal, h=hour. Relative expression normalized to GAPDH is shown. Error bars show SD from 2 wells of the same ESC line cultured in parallel.

(B) ChIP-qPCR for ETV5 binding on putative enhancers shown in Fig 7C-E. ChIP was performed in duplicate (1-2) on ETV5-C-3xFlag knock-in RGd2 cells using an anti-Flag antibody. Error bars indicate SD from 2 qPCR replicates.

(C) Western blot for POU3F1 and LEF1 in RGd2 ESC and *Pou3f1/Lef1* single and double knock-out clonal ESC lines. Black line indicates where two halves of the blot were merged after removal of two lanes from a mistargeted clone.

(D) GFP profiles in N2B27 at 26h post-2i withdrawal (N26h).

(E) RT-qPCR at 48h post-2i withdrawal. Error bars show SD from 2 qPCR replicates. Data from 2 independently derived *Lef1/Pou3f1*-dKO (LP-dKO) clonal lines are presented.

(F) UCSC Genome browser tracks showing normalized ChIP-seq read coverage for *Etv5* and H3K4me3 (this study), p300, H3K27Ac and H3K4me1 (Buecker *et al* , 2014)

(G) RT-qPCR for Oct4 in 2iLIF and EpiLC on RGd2 and *Etv5*-KO ESCs (2 independent clonal lines). Error bars show SD from 2 technical replicates for qPCR.

(H) Mean read coverage for p300, H3K27Ac and H3Kme1 (from Buecker *et al* , 2014) on promoter-associated ETV5-bound loci. Read depth scaled to 1x.

Supplemental Tables

Supplemental Table S1. Differentially expressed genes in *Rbpj* mutants and intersection with genes repressed by RBPJ and TCF3 (Related to Figures 2 and S4)

Supplemental Table S2. Expression of all genes in the RNA-seq datasets (Related to Figure 5)

Supplemental Table S3. Differentially expressed genes in ETV5-KO Np5 vs. RGd2 2ip5 (Related to Figure 5)

Supplemental Table S4. Summary of phenotypes in chimeric embryos (Related to figure 6)

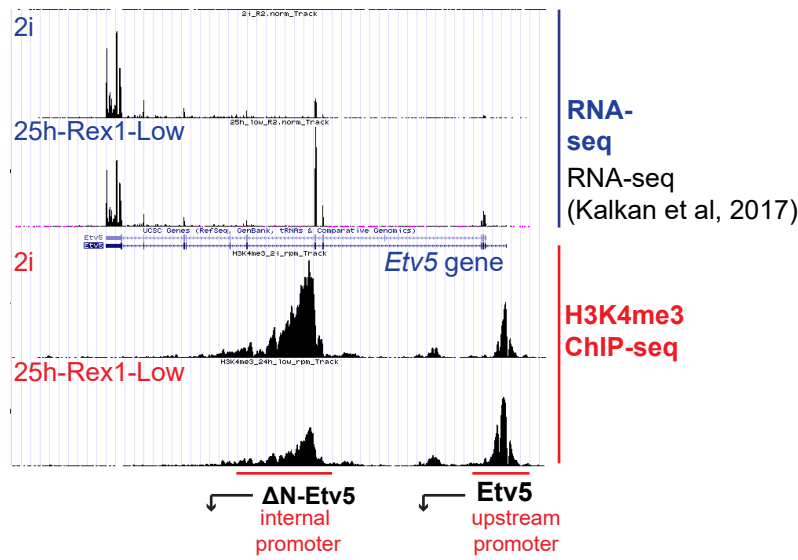
Supplemental Table S5. ETV5-associated genomic loci (Related to Figure 7)

Supplemental Table S6. Differentially expressed genes in *Etv5* mutants and intersection with genes regulated by ETV5, RBPJ and TCF3 (Related to Figures 7 and S6)

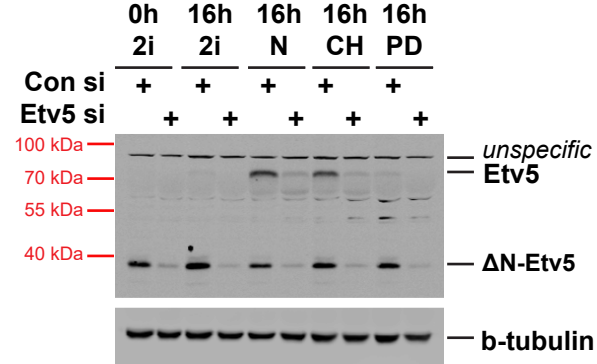
Supplemental Table S7. Reagents (Related to STAR Methods)

Figure S1 (Related to Figure 1)

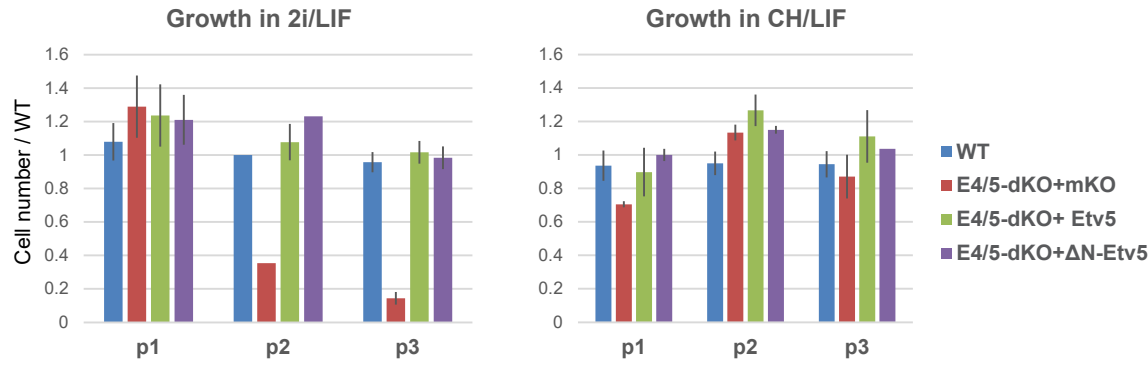
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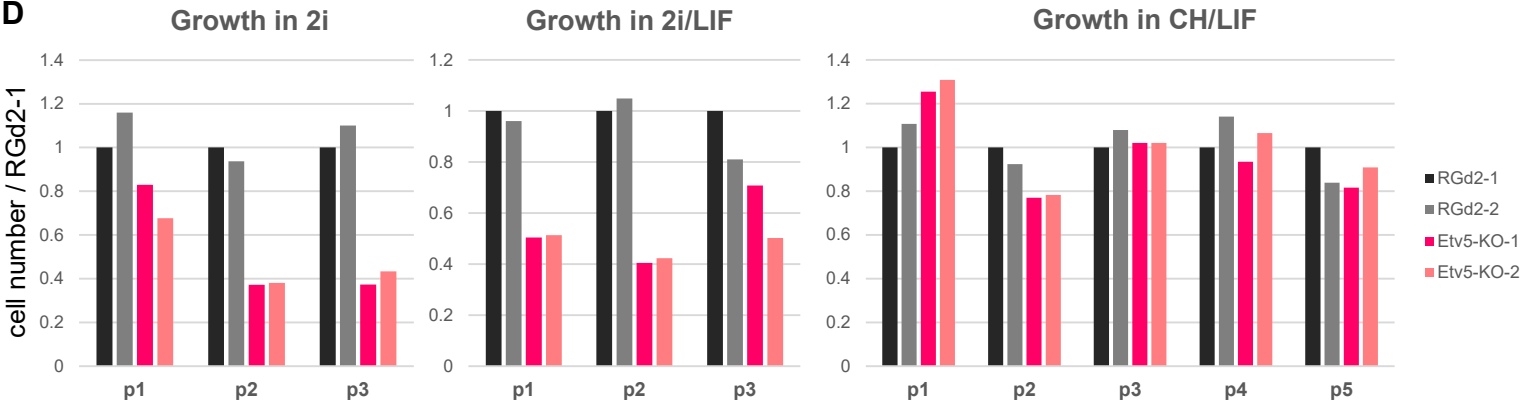
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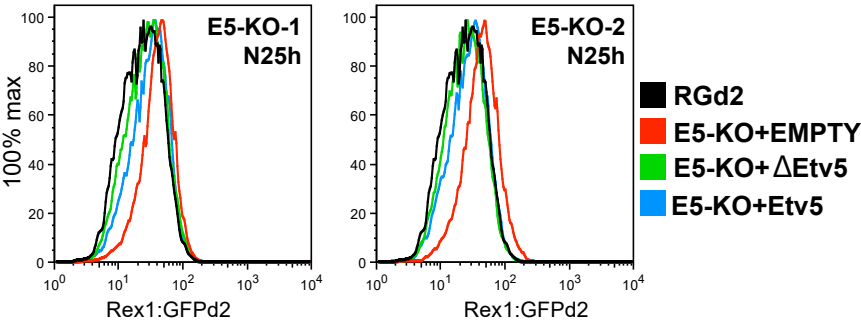
C



D



E



F

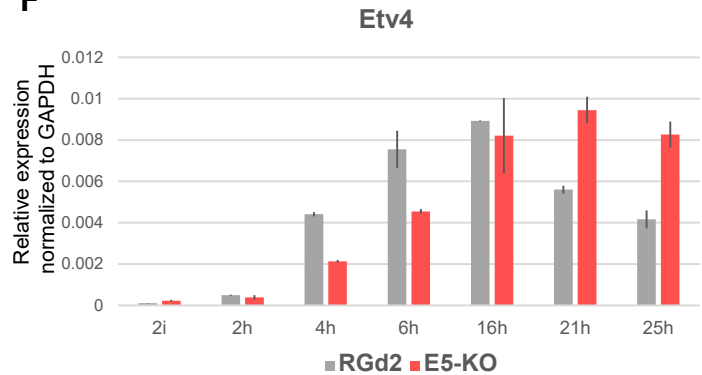


Figure S2 (Related to Figures 1 and 2)

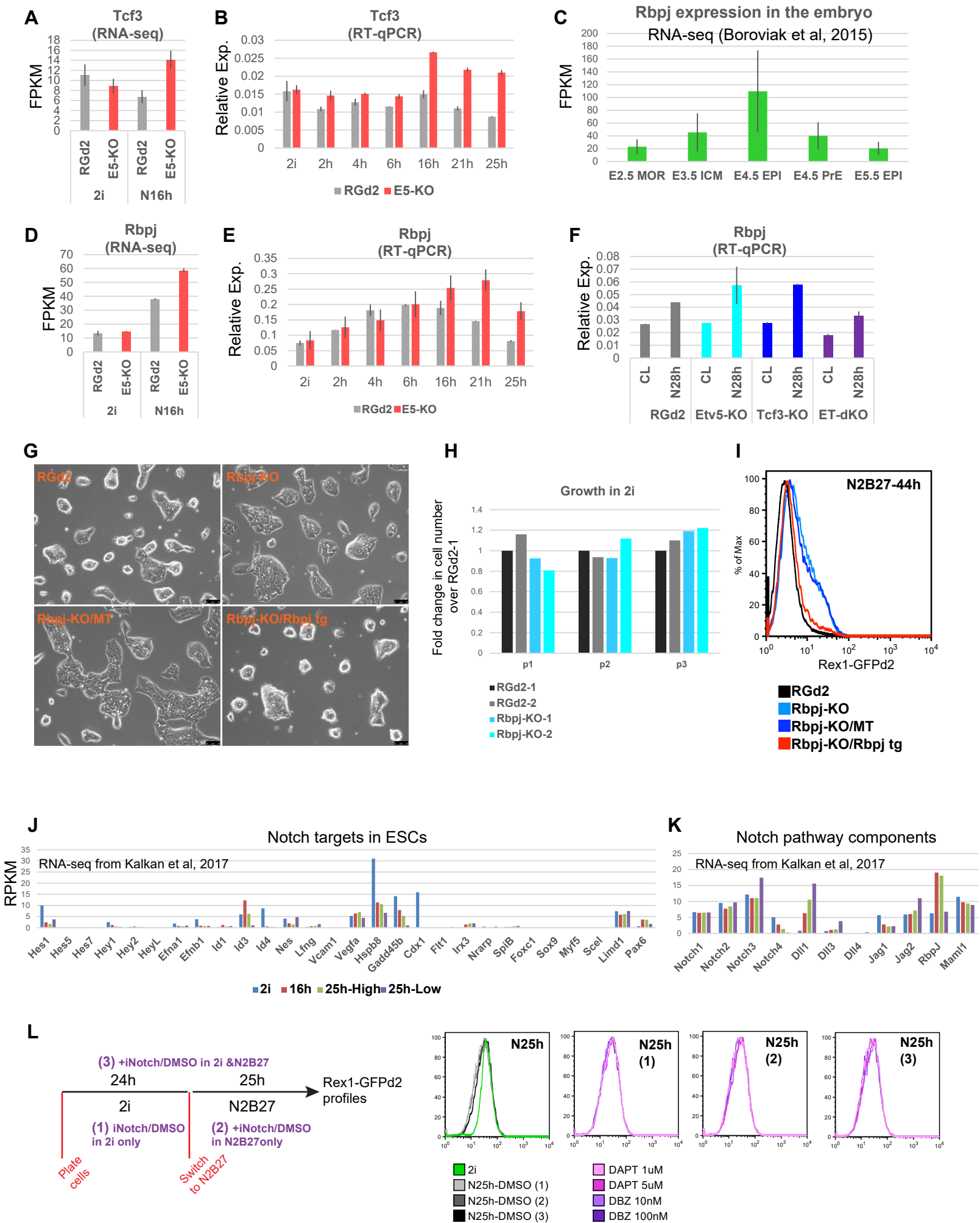


Figure S3 (Related to Figure 2)

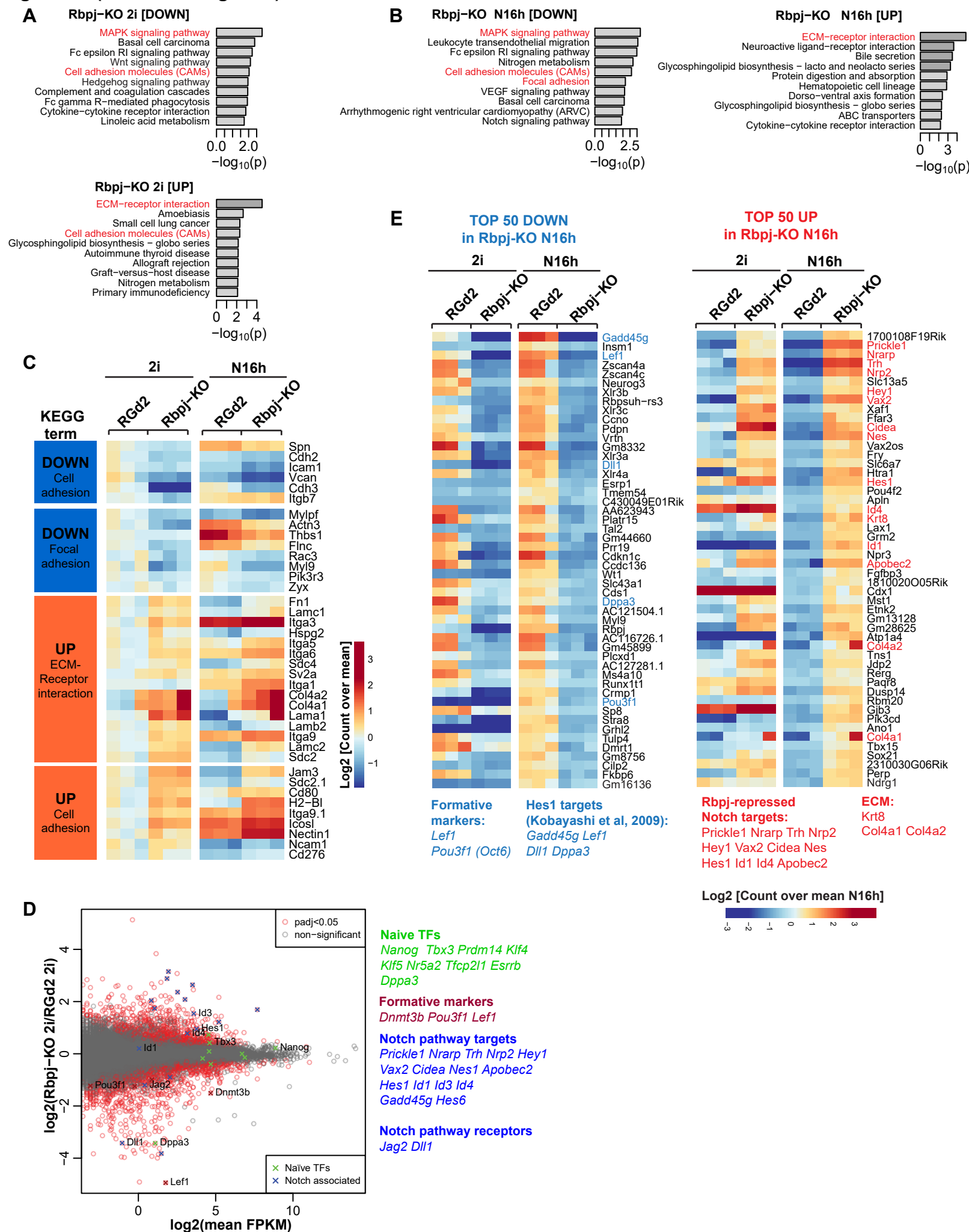


Figure S4 (Related to Figures 2 and 3)

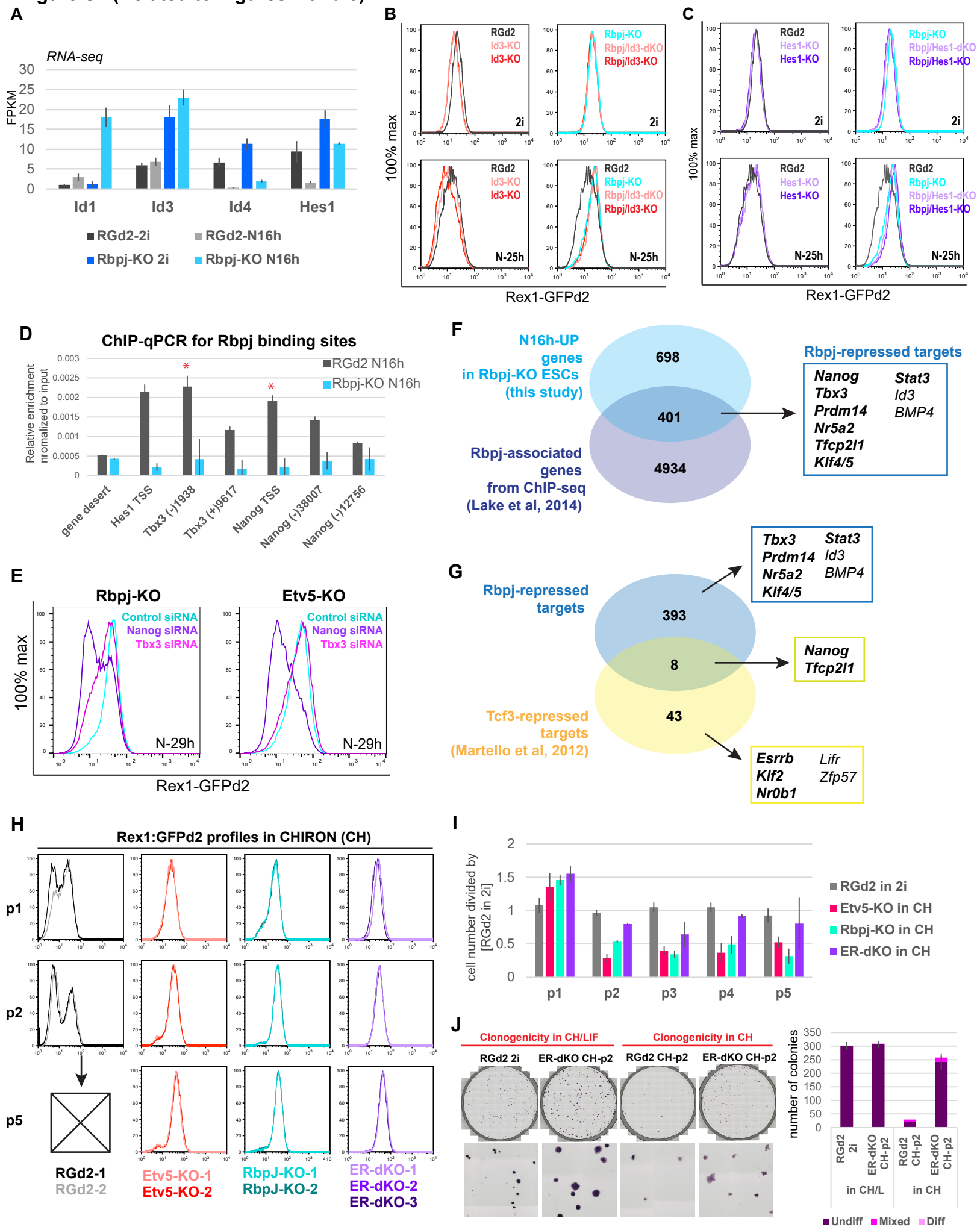
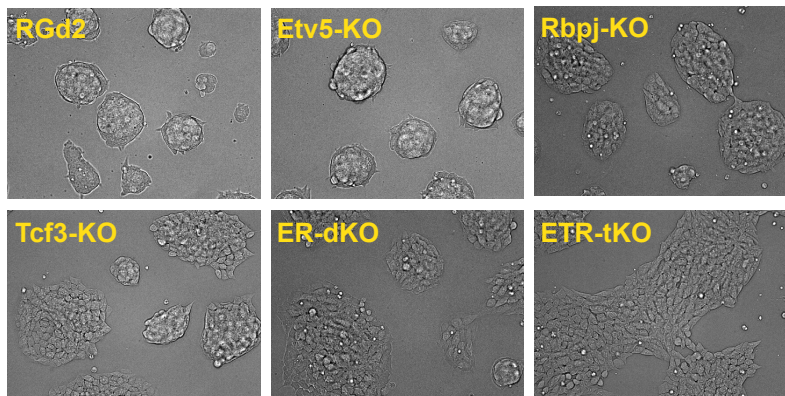
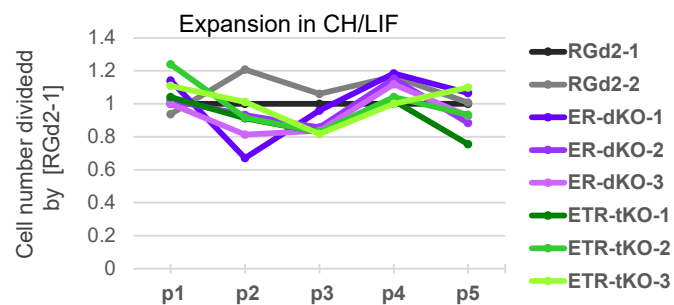


Figure S5 (Related to Figure 3)

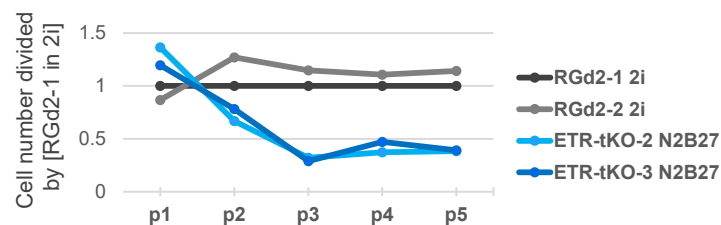
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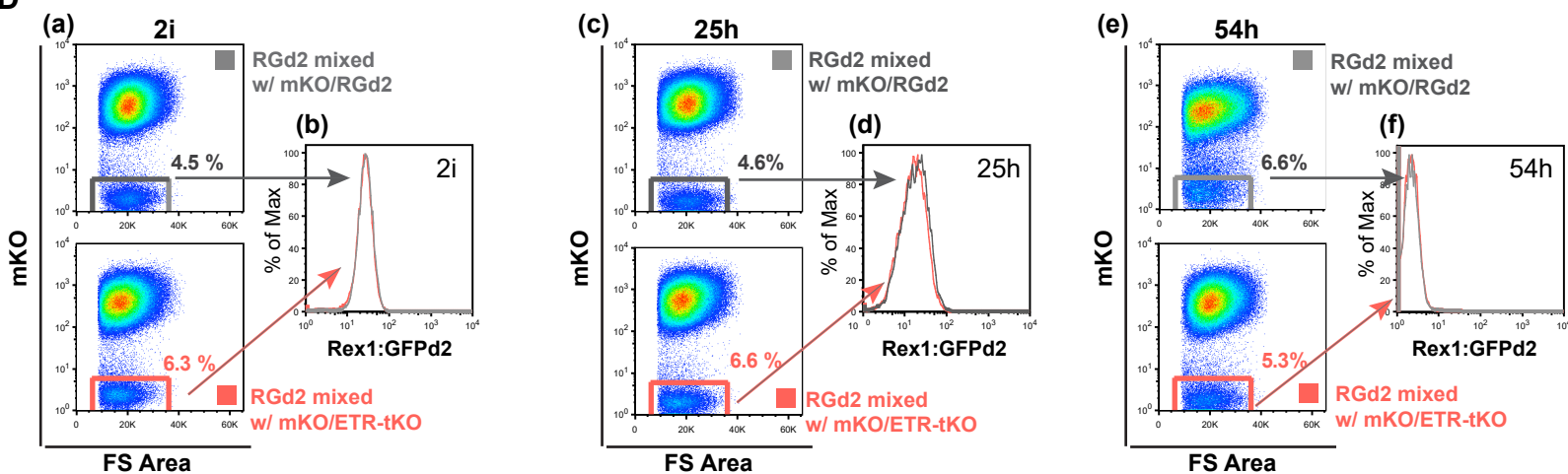
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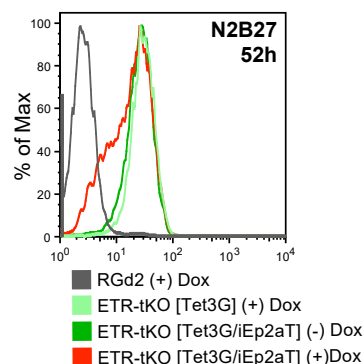
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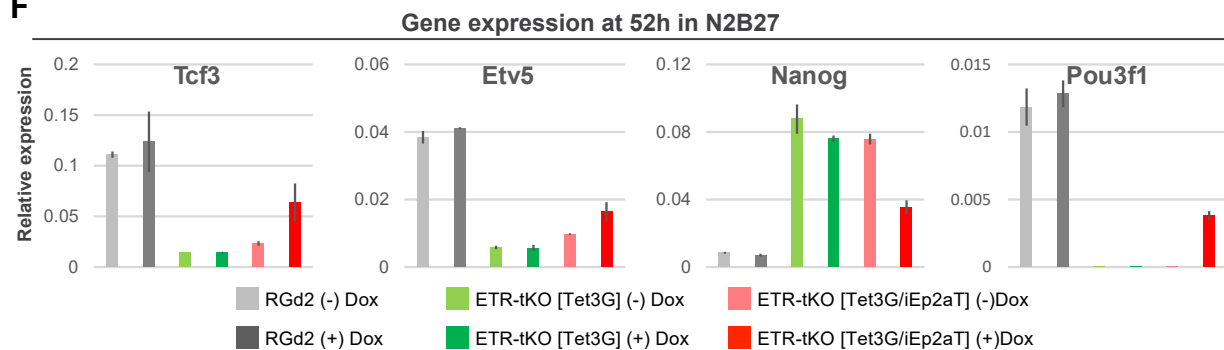
D



E



F



G

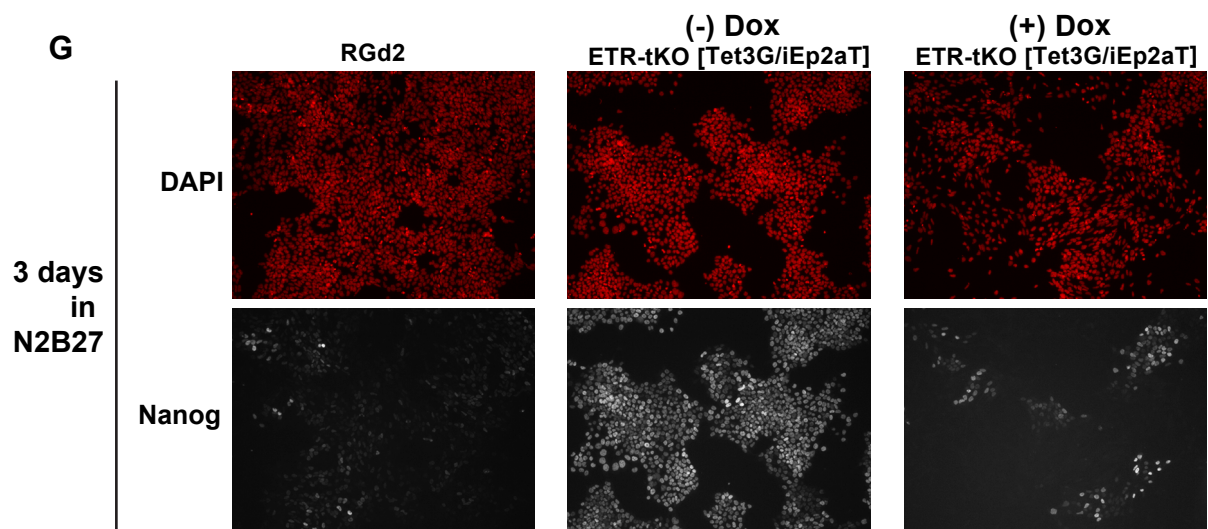


Figure S6 (Related to Figure 7)

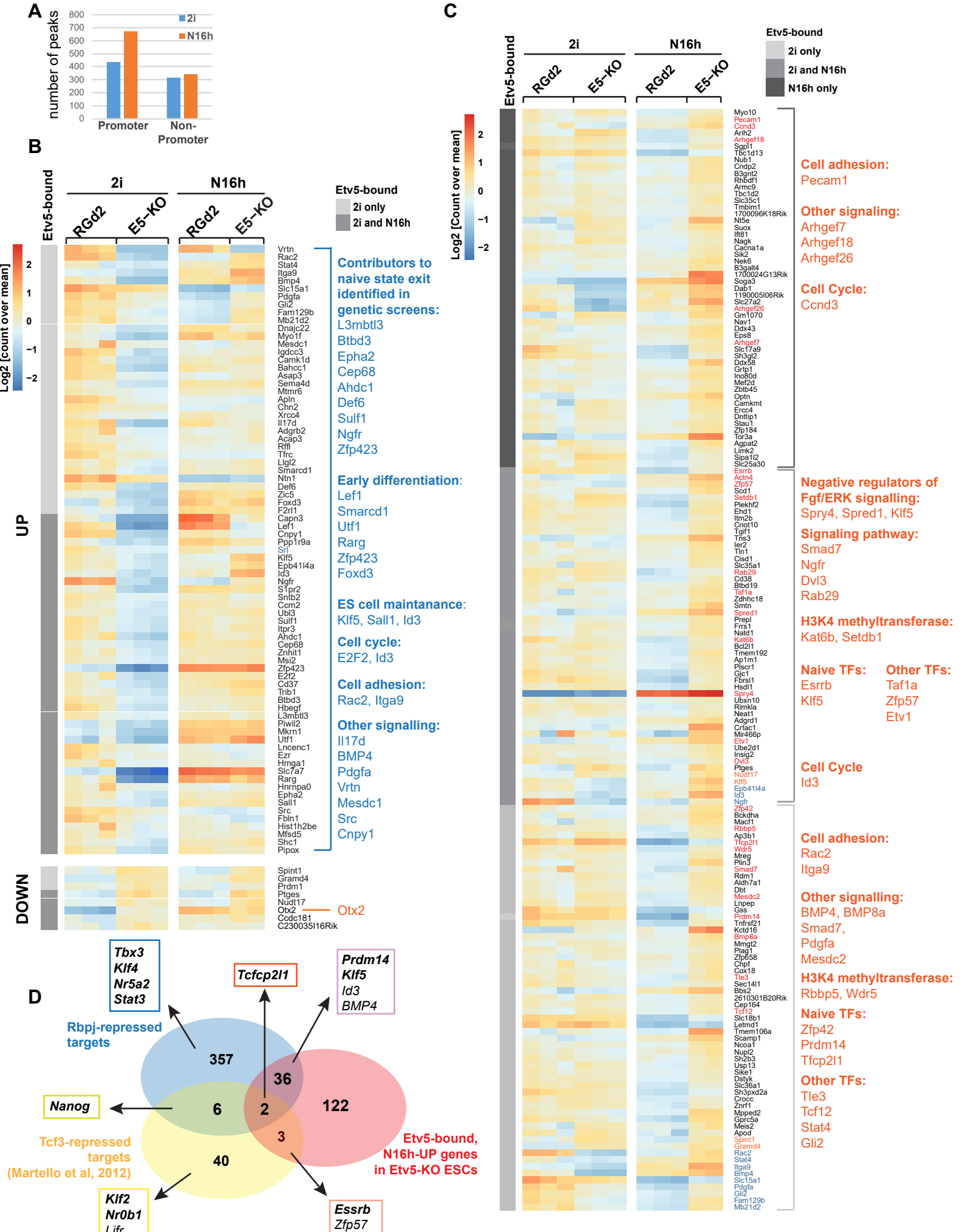


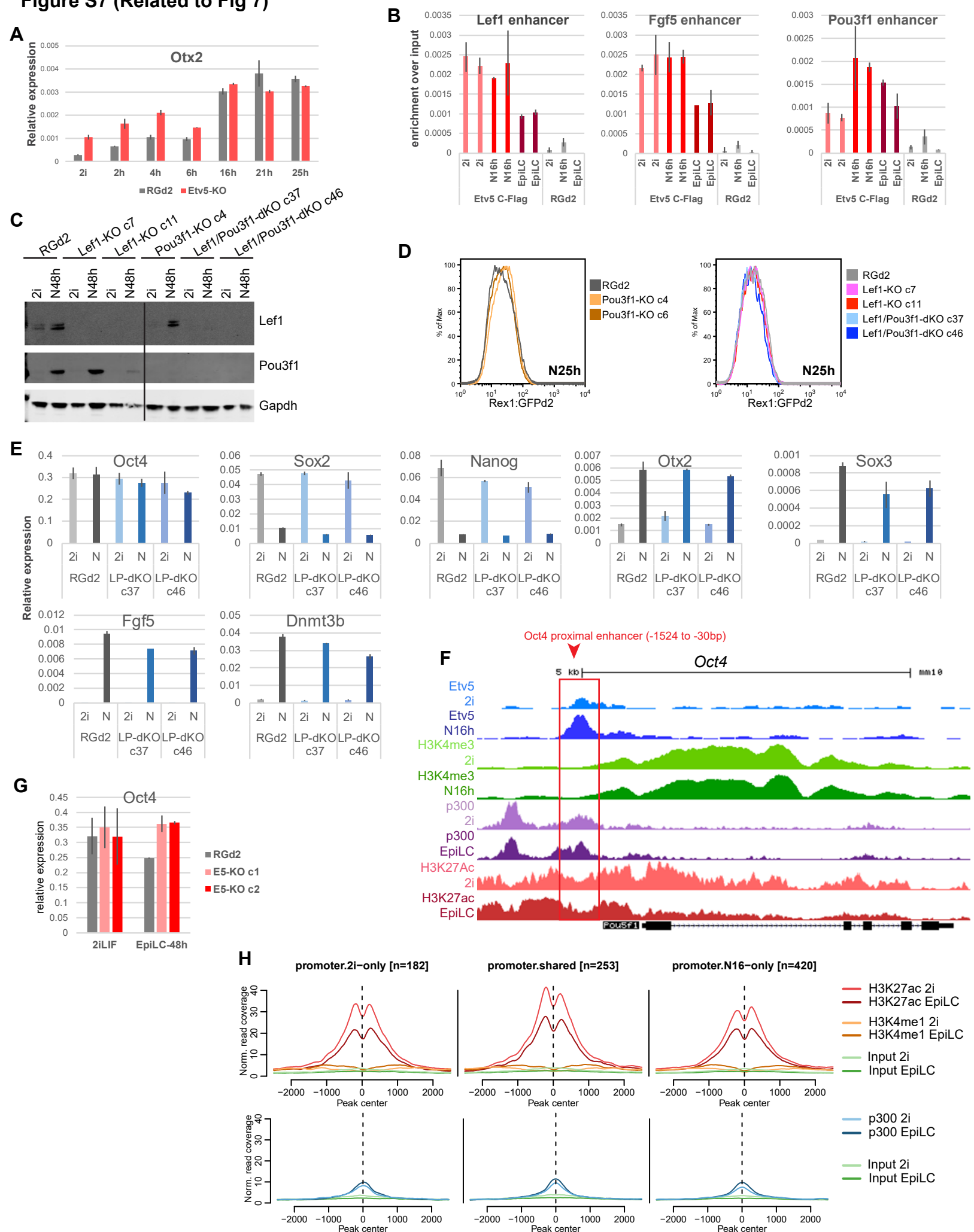
Figure S7 (Related to Fig 7)

Table S4 Classification of chimaeras


Stage analysed	Injected cell genotype	Embryos injected	Embryos transferred (recipients)	Embryos recovered	Chimeras	Description / Classification
E4.5	WT	5	NA	NA	100% (5/5)	High contribution exclusively to ICM
	KO	9	NA	NA	100% (9/9)	High contribution exclusively to ICM
E6.5	WT	5	5 (1)	100 % (5/5)	100% (5/5)	High contribution to epiblast (5/5); None Rex1 positive; All with posterior T expression; Normal: 5/5 Retarded: 0/5 Abnormal: 0/5
	KO	10	9 (1)	77.78% (7/9)	100% (7/7)	Moderate contribution to epiblast (7/7); Rex1 signal in majority of injected cells (7/7); T expression (1/7, weak); Contribution extending across embryonic/extraembryonic boundary (4/7); Normal: 0/7 Retarded: 4/7 Abnormal: 3/7
E7.5	WT	16	16 (2)	100 % (8/8) 100% (8/8)	81.25% (13/16)	High contribution of injected cells (13/13); No Nanog signal detected in 16/16 embryos; Pou3f1 signal in anterior epiblast (8/8); T signal in the primitive stream region (8/8); Normal: 16/16 Retarded: 0/16 Abnormal: 0/16
	KO	44	44 (4)	0% (0/11) 0% (0/11) 54.55% (6/11) 63.64% (7/11)	100% (13/13)	Recipient failure for 2/4 hosts; Mild to moderate contribution of injected cells (13/13); Detached Reichert's membrane (10/13); High Nanog in majority of injected cells (13/13); scattered Pou6f1 signal in the epiblast (7/7), but not in injected cells; T expression (0/6); Normal: 0/13 Retarded: 0/13 Abnormal: 13/13

Normal: No apparent developmental defects

Abnormal: Malformed embryos

Retarded: Small, but normal morphology

All chimaeras were generated by microinjection of 8 cells into E2.5 embryos.

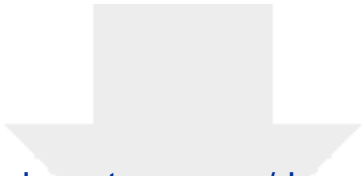


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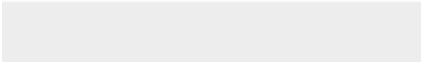
TableS1_DEG_Rbpj_KO_vs_RGd2_and_Rbpj_Tcf3_targ
ets.xlsx





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TableS2_All_RNAseq_unfiltered.xlsx





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TableS3_DEG_ETR_Np5_vs_RGd2_2ip5.xlsx






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TableS5_Etv5_ChIPseq_loci_peak_to_genes.xlsx





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TableS6_DEG_Etv5_KO_vs_RGd2_and_Etv5_Rbpj_Tcf3
_targets.xlsx





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TableS7_reagents_Related_to_STARMETHODS.xlsx



Supplemental Figures

Figure S1. Etv4/5 expression and function (Related to Figure 1)

Figure S2. Rbpj expression and function (Related to Figures 1 and 2)

Figure S3. Rbpj expression and function (Related to Figure 2)

Figure S4. Rbpj expression and function (Related to Figure 2)

Figure S5. Dual and triple knock out phenotypes (Related to Figure 3)

Figure S6. Etv5 association with transcriptionally dynamic promoters and enhancers (Related to Figure 7)

Figure S7. Etv5 association with transcriptionally dynamic promoters and enhancers (Related to Figure 7)

Supplemental Table

Table S4. Summary of phenotypes in chimeric embryos (Related to figure 6)

Supplemental Excel Spreadsheets

Supplemental Table S1. Differentially expressed genes in *Rbpj* mutants and intersection with genes repressed by RBPJ and TCF3 (Related to Figures 2 and S4)

Supplemental Table S2. Expression of all genes (Related to Figure 5)

Supplemental Table S3. Differentially expressed genes in ETR-KO Np5 (Related to Figure 5)

Supplemental Table S5. ETV5-associated genomic loci (Related to Figure 7)

Supplemental Table S6. Differentially expressed genes in *Etv5* mutants and intersection with genes regulated by ETV5, RBPJ and TCF3 (Related to Figures 7 and S6)

Supplemental Table S7. Reagents (Related to STAR Methods)